

(19) World Intellectual Property Organization  
International Bureau



(43) International Publication Date  
26 July 2007 (26.07.2007)

PCT

(10) International Publication Number  
**WO 2007/084954 A2**

(51) International Patent Classification:  
C12N 15/09 (2006.01)

(21) International Application Number:  
PCT/US2007/060712

(22) International Filing Date: 18 January 2007 (18.01.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/760,321 19 January 2006 (19.01.2006) US

(71) Applicant (for all designated States except US): **THE BOARD OF TRUSTEES OF THE UNIVERSITY OF ILLINOIS** [US/US]; 352 Henry Administration Building, 506 South Wright Street, Urbana, IL 61801 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **PRABHAKAR, Bellur, S.** [US/US]; 511 St. Johns Court, Oakbrook, IL 60523 (US). **MULHERKAR, Nirupama** [IN/US]; 1580 Pelham Parkway South #5k, Bronx, NY 10461 (US).

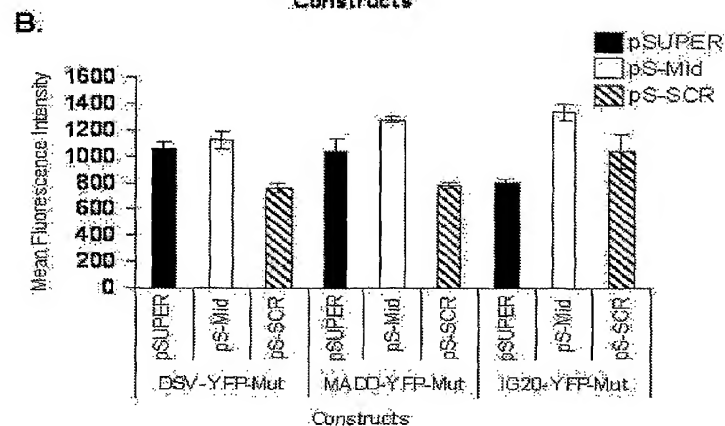
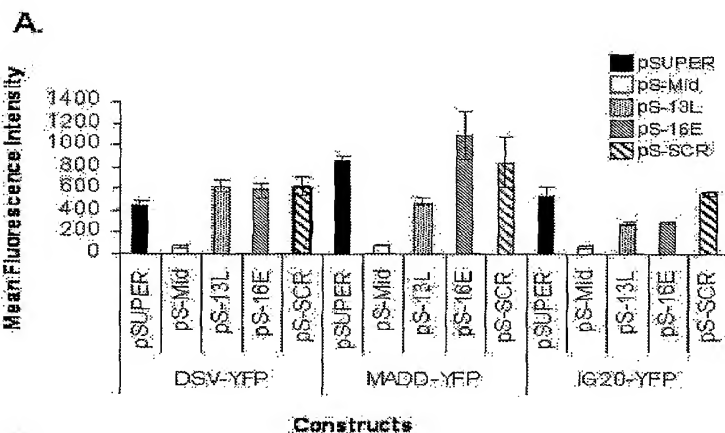
(74) Agents: **DEVADAS, Sendil et al.**; Barnes and Thornburg LLP, P.o. Box 2786, Chicago, IL 60690-2786 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LV, LY, MA, MD, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

[Continued on next page]

(54) Title: SELECTIVE INHIBITION OF IG20 SPLICE VARIANTS TO TREAT CANCERS



(57) Abstract: Methods and compositions inhibit the growth of cancer cells by selectively down-regulating the expression of an IG20 splice variant including MADD. Specific knock-down of MADD splice variant resulted in the apoptosis of cancer cells. Interfering RNAs including small hairpin RNAs (shRNA) to down-regulate MADD expression in vivo are disclosed.



**Published:**

— without international search report and to be republished  
upon receipt of that report

*For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

## SELECTIVE INHIBITION OF IG20 SPLICE VARIANTS TO TREAT CANCERS

### BACKGROUND

- [0001] In eukaryotes, many genes undergo alternative splicing and encode multiple isoforms leading to the expression of related proteins that have distinct biochemical as well as biological features. The *IG20* (Insulinoma-Glucagonoma) is one such gene that undergoes alternative splicing and encodes at least four different splice variants (SVs), namely IG20pa, MADD/DENN, IG20-SV2 and DENN-SV. These four *IG20*-SVs are distinguished by differential splicing of exons 13L and 16. Upon comparison to IG20pa, the splice variants MADD, IG20-SV2 and DENN-SV lack the expression of exon 16 or 13L, or both respectively. All four *IG20*-SVs express an N-terminal leucine zipper and a C-terminal death-domain homology region.
- [0002] The *IG20* gene plays an important role in cancer cell proliferation, apoptosis and survival, most likely through its effects on MAP kinase activation and other cell signaling pathways. Additionally, it plays an important role in neurotransmission, neurodegeneration and guanine nucleotide exchange. How *IG20* is involved in these divergent functions is not yet full known.
- [0003] Expression of the *IG20* gene is relatively high in cancer cells and tissues as compared to the levels of expression in their normal counterparts. While MADD and DENN-SV are constitutively expressed (DENN-SV is over-expressed relative to other SVs in cancer), expression of IG20pa and IG20-SV2 appears to be regulated in that they may or may not be expressed in certain cells. Gain of function studies through expression of individual *IG20*-SVs in HeLa cells showed that MADD and IG20-SV2 variants have little or no effect on cell proliferation and induced apoptosis. IG20pa increased susceptibility to both extrinsic and intrinsic apoptotic stimuli, and suppressed cell proliferation and DENN-SV conferred resistance to induced apoptosis and enhanced cell proliferation. Thus IG20pa and DENN-SV acted like a “tumor suppressor” and an “oncogene” respectively.
- [0004] Knock-down of all endogenous *IG20*-SVs, using anti-sense oligonucleotides, resulted in spontaneous apoptosis of cancer cells *in vitro* and *in vivo*, but not normal cells. Since different splice variants have different functions and the function of *IG20* gene may vary depending upon the cell type, it is prudent to develop modalities that allow knockdown of specific isoforms to achieve the desired effect including altering cell growth, apoptosis, neuron-transmission. Such isoform-specific knock-down has not yet been demonstrated. In addition, the contrasting effects of *IG20*-SVs noted from gain of function studies can be clarified by knocking-down individual endogenous *IG20*-SVs and determining the consequent effects. This poses several challenges because various *IG20*-SVs differ from each other only by the differential expression of very short exons 13L (130 base pairs) and 16 (60 base pairs). Therefore, knock-down of specific splice variants of *IG20* gene is difficult because of availability of very short target sequences that are

differentially expressed in different splice variants and is achieved through the use of specially designed small hairpin RNA molecules (shRNA) disclosed herein.

#### SUMMARY

- [0005]** Methods and compositions selectively down-regulate the expression of a particular *IG20* gene splice variant and thereby promoting cancer cell death in cells that express the particular splice variant. For example, MADD splice variant of *IG20* gene was down regulated by the use of interfering RNA sequences that specifically down regulate MADD splice variant and the down regulation was sufficient to cause apoptotic death of cancer cells.
- [0006]** Using shRNAs that specifically target exon 15 that is expressed in all isoforms of *IG20* and designated Mid, or exons 13L and 16 that are differentially expressed in *IG20*-SVs, the various splice variants of *IG20* gene were selectively knocked-down in HeLa and PA-1 cells. Knock down of MADD (one of the *IG20* splice variants) resulted in spontaneous apoptosis and this effect is reversible by re-expressing the MADD protein. This result indicated that MADD is required and sufficient for the survival of cancer cells. Further, knock down of MADD rendered cells more susceptible to TRAIL induced apoptosis. The increased apoptosis was associated with increased caspase-8 and caspase-3 activation. The results presented here demonstrate that knock-down of MADD causes a significant increase in spontaneous as well as TRAIL induced cell death of cancer cells, and support the notion that it is MADD, and not the other three isoforms (*i.e.* DENN-SV, *IG20*-SV2 and *IG20*) that is required for cancer cell survival.
- [0007]** A method of selectively inhibiting a splice variant of an *IG20* (Insulinoma-Glucagonoma) gene, the method includes the steps of:
- (a) obtaining an agent that selectively inhibits the expression of an *IG20* splice variant; and
  - (b) contacting a cell that expresses the splice variant to inhibit the splice variant.
- [0008]** A suitable splice variant is MADD and a suitable agent is a molecule selected from the group that includes siRNA, shRNA and an anti-sense molecule against the *IG20* splice variant.
- [0009]** A method of specifically down-regulating the expression of a splice variant of an *IG20* (Insulinoma-Glucagonoma) gene, the method includes the steps of:
- (a) obtaining a nucleic acid molecule, wherein the nucleic acid molecule or a transcription product thereof is capable of selectively binding to a RNA molecule, the RNA molecule including a nucleic acid sequence of a MADD splice variant of the *IG20* gene; and
  - (b) contacting a cell that expresses the MADD splice variant of the *IG20* gene with the nucleic acid molecule, wherein the nucleic acid molecule down-regulates the expression of the MADD splice variant.

- [00010] A nucleic acid molecule includes a nucleotide sequence of CGGCGAATCTATGACAATC or a transcribed product thereof that is sufficient to knock-down the expression of MADD splice variant or an allelic variant or a mutant thereof.
- [00011] A method of inhibiting the growth of a cancer cell includes the steps of:
- (a) obtaining a nucleic acid molecule, wherein the nucleic acid molecule is capable of selectively binding to a RNA molecule of a MADD splice variant of the *IG20* gene; and
  - (b) contacting a cancer cell that expresses the MADD splice variant of the *IG20* gene with the nucleic acid molecule, wherein the nucleic acid molecule down-regulates the expression of the MADD splice variant.
- [00012] A suitable nucleic acid molecule targets exon 13L of the MADD splice variant.
- [00013] A suitable nucleic acid molecule is selected from a group that includes siRNA, shRNA and anti-sense molecule against the MADD splice variant of *IG20* that targets a nucleotide sequence of MADD selected from a group that includes CGGCGAATCTATGACAATC, allelic variation thereof, polymorphisms thereof, and a genetic mutation thereof.
- [00014] A method of regulating the growth of a cancer cell includes the steps of:
- (a) administering a pharmaceutical composition that consists essentially of a nucleic acid that specifically down-regulates the expression of a MADD splice variant of the *IG20* gene in a cancer cell; and
  - (b) exposing the cancer cell to a cancer treatment selected from the group consisting of radiation therapy, chemotherapy, and antibody therapy or a combination thereof.
- [00015] A method of increasing apoptotic cell death in a cancer cell includes the steps of:
- (a) administering a nucleic acid molecule capable of specifically down-regulating the expression of a MADD splice variant of an *IG20* gene in a cancer cell; and
  - (b) increasing the apoptotic cell death in the cancer cell.
- [00016] In an aspect, the apoptotic cell death is effected by a caspase. The apoptotic cell death is also induced by a TNF-related apoptosis inducing ligand (TRAIL).
- [00017] A suitable nucleic acid molecule capable of inducing apoptotic cell death includes a nucleotide sequence CGGCGAATCTATGACAATC or an RNA equivalent thereof.
- [00018] A pharmaceutical composition consists essentially of a nucleic acid sequence capable of selectively inhibiting the expression of a MADD splice variant in a cancer cell. The pharmaceutical composition includes a nucleic acid whose nucleotide sequence includes CGGCGAATCTATGACAATC or an RNA equivalent thereof.
- [00019] A vector includes a nucleic acid sequence capable of selectively inhibiting the expression of a MADD splice variant in a cancer cell, wherein the nucleic acid sequence includes CGGCGAATCTATGACAATC.

- [00020] A cell includes an exogenous molecule of an interfering RNA, wherein the RNA molecule specifically down-regulates the expression of a MADD splice variant of an *IG20* gene. The cell may be a cancer cell or a cell predisposed or likely to become cancerous.
- [00021] An isolated nucleic acid molecule encodes a short interfering RNA, the nucleic acid includes the structure:
- $$X_{\text{sense}}\text{---hairpin loop---}X_{\text{anti-sense}}$$
- [00022] wherein X includes a nucleic acid sequence CGGCGAATCTATGACAATC. A suitable shRNA sequence is generated by a nucleic acid sequence CGGCGAATCTATGACAATCTTCAAGAGAGATTGTCATAGATTTCGCCG, wherein the hairpin loop region is from positions 20-28 (shown as underlined). The nucleic acids may be synthetic.
- [00023] An isolated RNA molecule includes a nucleic acid sequence CGGCGAAUCUAUGACAAUC.

#### BRIEF DESCRIPTION OF THE DRAWINGS

- [00024] **FIG. 1** shows screening of shRNAs for *IG20* knock-down. **A.** Knock-down of expression of exogenous *IG20*-SVs. 293T cells were plated onto 12-well plates and cotransfected with the indicated *IG20*-SV-YFP and pSUPER-shRNA constructs. YFP expression is represented as Mean Fluorescence Intensity. Data shown (mean + SD of triplicates) are representative of three independent experiments each with P values <0.005 for all test groups. **B.** Specificity of Mid-shRNA: Shows the inability of Mid-shRNA to suppress the expression of mutant-*IG20*pa, -MADD, and -DENN-SV constructs.
- [00025] **FIG. 2** shows differential down-modulation of endogenous *IG20*-SVs in HeLa cells. 1 µg total RNA obtained from HeLa cells at 72 hours post-transduction was used for RT-PCR. The products were separated on a 2% agarose gel. **(A)** Shows amplification of all four *IG20*-SVs using F2-B2 primers. **(B)** Quantification of relative intensities of bands from panel-A using an Alpha Imager (Alpha Innotech Corporation, CA). **(C)** Amplification of *IG20*pa and MADD using 13L-Forward and B2-Reverse primers. **(D)** Quantification of relative intensities of bands from panel-C.
- [00026] **FIG. 3** illustrates effect of down-modulation of *IG20*-SVs in HeLa cells. **(A)** Summary of results showing percentage of cells with increased nuclear condensation as measured by Hoechst staining from three independent experiments. The P value was <0.005 for all test groups. Hoechst Staining- 72 hours post-transduction was performed and HeLa cells were collected and stained with 1 µg/mL of Hoechst and subjected to FACS analysis. Percentages of highly positive cells (apoptotic cells) are indicated on the histograms. **(B)** Summary of results showing percentage of cells with increased mitochondrial depolarization from three independent experiments. The P value was <0.005 for all test groups. The data were collected from only GFP positive cells. Mitochondrial Depolarization-72 hours post-transduction was performed, HeLa cells were collected, and stained with TMRM. Loss of staining as a marker of mitochondrial depolarization was detected by FACS.

- [00027] **FIG. 4** shows activation of caspases upon down-modulation of *IG20*-SVs. (A) General activation of caspases-60 hours post-transduction, HeLa cells were collected and stained for all activated caspases using Red-z-VAD-FMK; (B) for Caspase-8 with RedIETD-FMK; and (C) for Caspase-9 with Red-LEHD-FMK, and analyzed by FACS. Percentage caspase activation in GFP-positive cells is shown (Mean + SD of triplicates).
- [00028] **FIG. 5** shows effects of *IG20* down-modulation on HeLa cell proliferation. Cell Growth. 24 hours post-transduction, HeLa cells were plated as described in materials and methods. Cells were then harvested and viable cells (trypan blue negative cells) were counted on indicated days. Data represent Mean + SD of triplicates. Cell proliferation. 24 hours post-transduction HeLa cells were stained with CF SE-red (SNARF-1carboxylic acid, acetate, succinimidyl ester), harvested on indicated days and evaluated for CFSE-dilution by FACS. The numbers on the histograms indicate geometric peak mean intensities of CFSE staining in the transduced cells. Cell survival. Show crystal violet staining of cells surviving upon *IG20* down-modulation.
- [00029] **FIG. 6** shows effects of *IG20* down-modulation in PA-1 cells. (A) Down-modulation of endogenous *IG20* in PA-1 cells. RT-PCR of *IG20*-SVs using F2-B2 primers from PA-1 cells 72 hours post-transduction. (B) Amplification of only *IG20*pa and MADD using 13L-F and B2 primers. Nuclear Condensation was also determined. 72 hours post-transduction, PA-1 cells were stained with Hoechst stain and analyzed by FACS. Percentages of apoptotic cells are indicated on the histograms. Mitochondrial Depolarization was performed. 72 hours post transduction, PA-1 cells were stained with TMRM and analyzed by FACS. Data shown are representative of three different experiments.
- [00030] **FIG. 7** shows effects of *IG20* down-modulation on PA-1 cell proliferation. (A) Cell Growth (B) Proliferation (C) Cell Survival. Experiments were carried out as described previously with HeLa cells (see FIG. 5 legend). Data shown are representative of three different experiments.
- [00031] **FIG. 8** shows MADD down-modulation in HeLa and PA-1 cells results in spontaneous apoptosis. (A) and (C) show RT-PCR profile of *IG20*-SVs at 24, 48 and 72 hours post shRNA-transduction in HeLa and PA-1 cells respectively. (B) and (D) show spontaneous apoptosis as measured by Hoechst staining in transduced HeLa and PA-1 cells respectively. Data shown represent Mean  $\pm$  SD of triplicates.
- [00032] **FIG. 9** represents down-modulation of MADD enhances susceptibility to TRAIL. Kinetics of response (A) and (C). Thirty-six hours post-transduction, HeLa cells were treated with 50 ng of TRAIL for different durations. Dose response (B) and (D). HeLa cells were treated for five hours with the indicated concentrations of TRAIL. (A and B) Active-caspase-3 was detected using a PE-conjugated antibody specific for active-caspase-3 by FACS. Data presented are representative of three different experiments and the P-value was  $<0.005$ . (C and D) Apoptosis was also measured by TMRM exclusion using FACS. Data shown represent mean  $\pm$  SD of triplicates.

- [00033] **FIG. 10** illustrates down-modulation of MADD results in caspase-8 activation. (A) HeLa cells and (B) PA-1 cells were collected 48h post-transduction and probed for caspase-8 and FADD which are components of the DISC. As observed, the cleaved intermediate fragments of caspase-8 (early indication of caspase-8 activation) can be detected upon MADD abrogation. These results were reproduced thrice.
- [00034] **FIG. 11** represents enhanced TRAIL-mediated apoptosis results from increased caspase-8 activation. TRAIL treatment results in recruitment of pro-caspase-8 to the receptors followed by its cleavage resulting in its activation. The cleaved intermediate fragments (p43/p41) and the catalytically active subunits of caspase-8 (p18) were detected upon TRAIL treatment. Caspase-8 is the initiator caspase that can cause activation of the effector caspase-3. Decrease in the amount of pro-caspase-3 indicates its cleavage to form active-caspase-3. Actin levels serve as loading control.
- [00035] **FIG. 12** shows that CrmA and DN-FADD inhibit the onset of spontaneous apoptosis. (A) HeLa and (B) PA-1 cells stably expressing control vector, CrmA or DN-FADD were transduced with SCR, Mid and 13L shRNAs. Spontaneous apoptosis was assessed by Hoechst staining 72h post-transduction. Data shown are representative of three different experiments. P-value in each case was <0.005.
- [00036] **FIG. 13** shows DN-FADD and CrmA inhibit TRAIL-induced apoptosis. HeLa cells stably expressing control vector, CrmA or DN-FADD were transduced with SCR, Mid and 13L shRNAs. Thirty-six hours post-transduction, cells were treated with 50ng of TRAIL for 5 hours. Apoptosis was measured by active-caspase-3 staining. Data shown are representative of three different experiments. Error bars indicate mean  $\pm$  SD of triplicates.
- [00037] **FIG. 14** shows the results of immunoprecipitation of DR4/DR5 in the absence of ligand. Forty-eight hours transduced (A) HeLa cells and (B) PA-1 cells ( $1 \times 10^7$ ) were collected and lysed. Lysates were normalized for protein concentration and DR4s/DR5s were immunoprecipitated. Separated immune complexes were immunoblotted using antibodies specific for caspase-8, FADD and DR4/DR5. The caspase-8 and FADD, and DR4/DR5 immunoblots were exposed for 4 hours and 30 minutes respectively. This is a representative of three different experiments.
- [00038] **FIG. 15** shows down-modulation of MADD results in increased caspase-8 activation at the TRAIL DISC. The TRAIL DISC was immunoprecipitated using a biotinylated-antibody specific for TRAIL from HeLa cells that were either left untreated or treated with 250ng of TRAIL for 30 minutes. Complexes were separated on 12% SDS PAGE gel and subjected to immunoblotting with the indicated antibodies. All blots were similarly exposed to the film. Increased recruitment of FADD and pro-caspase-8 was observed after TRAIL treatment, but increased casapase-8 activation was more apparent upon MADD down-modulation.
- [00039] **FIG. 16** demonstrates that MADD exerts its anti-apoptotic effect by binding to DR4 but not other components of the DISC. To determine whether MADD prevents caspase-8 activation by directly binding to caspase-8, HeLa cells were transfected with MADD-YFP. Thirty-six hours post-



transduction, cells were either left untreated or treated with 250ng of TRAIL for 30 minutes. Samples were lysed and the lysates were normalized for protein concentration and pre-cleared. Equal amount of proteins were incubated with either anti-*IG20* or anti-caspase-8 antibody. Complexes were subjected to immunoblotting as described before. Results show MADD binding to DR4, but not caspase-8 or FADD.

**[00040]** FIG. 17 is an illustration of a shRNA vector used herein that is capable of stable expression of shRNAs.

**[00041]** FIG. 18 is a schematic illustration of the targets of some of the shRNAs used to knock-down specific *IG20* splice variants (isoforms).

**[00042]** FIG. 19 provides schematic illustration of screening for shRNAs (A) and lentivirus production that contain the shRNA expressing vectors (B).

**[00043]** FIG. 20 is a chart demonstrating the knock-down effects of the respective shRNAs directed against *IG20* splice variants, as measured by mean fluorescent intensity.

#### DETAILED DESCRIPTION

**[00044]** Methods and compositions relate selective down-regulation of a specific *IG20* splice variant to promote apoptosis of cancer cells. Through the use of specific small hairpin RNA (shRNA) molecules, knock-down of an *IG20* splice variant, e.g., MADD is demonstrated. This knock-down of MADD splice variant in cancer cells resulted in apoptotic cell death. Cell death of cancer cells is further characterized by activation of caspases that are responsible for apoptotic cell death.

**[00045]** Down-regulation of MADD splice variant can be accomplished by a number of ways. For example knock-down of MADD splice variant can be accomplished through shRNA, siRNA, anti-sense expression, small-molecules that specifically lower the RNA levels of MADD or inactivate the activity of MADD protein, and synthetic peptide nucleic acid (PNA) oligomers (e.g., containing repeating N-(2-aminoethyl)-glycine units linked by peptide bonds).

**[00046]** Agents capable of down-regulating MADD expression are delivered directly to tumors, administered by a viral vector capable of transcribing and producing an interfering RNA (RNAi) molecule, liposome, and as pharmaceutical compositions. shRNAs and siRNAs can also be delivered as synthetic molecules.

**[00047]** The *IG20* gene is over-expressed in human tumors and cancer cell lines, and encodes at least four splice variants (SVs) namely, *IG20* (here referred to as *IG20pa*), MADD, *IG20-SV2* and DENN-SV. Gain of function studies showed that *IG20*-SVs can exhibit diverse functions and play a critical role in cell proliferation and apoptosis. Expression of exogenous *IG20pa* or DENN-SV rendered cells either susceptible or resistant to induced apoptosis, respectively; while MADD and *IG20-SV2* had no apparent effect.

[00048] The contrasting effects of the *IG20*-SVs in a more physiologically relevant system are analyzed herein by using exon-specific shRNAs to selectively knock-down specific *IG20*-SVs. Knock-down of all *IG20*-SVs resulted in spontaneous apoptosis of HeLa and PA-1 cells. Simultaneous knock-down of all the splice variants of *IG20* may not be therapeutically as effective as selective knock-down because down-regulation of all the splice variants result in unexpected and undesirable outcomes because different splice variants exhibit different physiologically relevant functions such as cell growth, cell growth inhibition, neurotransmission and the like. Moreover, *IG20* gene through its splice variants, can exert different functional effects on different tissues (*e.g.* neurotransmission in neuronal cells). Also, knock down of all splice variants may be harmful as evidenced by the inability of *IG20* gene knockout mice to survive. Therefore, knock-down of select isoforms facilitates induction of the intended effect and minimizes harmful effects, *e.g.*, death of normal cells. Knock-down of MADD can render cells susceptible to spontaneous apoptosis but had no discernible effect on cell proliferation, colony size, or cell cycle progression. The utility of shRNAs for selective knock-down of particular *IG20*-SVs is demonstrated. MADD isoform expression is required for cancer cell survival, and therefore the methods and compositions disclosed herein are therapeutically valuable in targeting specific *IG20*-SVs to reduce cancer growth and thereby promoting selective cancer cell death.

[00049] MADD abrogation, in addition to causing spontaneous apoptosis, also enhances TRAIL-induced apoptosis. MADD interacts with the death receptors (DRs) but not with either the FADD or caspase-8, and the spontaneous as well as enhanced TRAIL induced apoptosis result from activation of caspase-8 at the DRs without an apparent increase in the recruitment of DISC components. Under physiological conditions, MADD acts as a negative regulator of caspase-8 activation.

[00050] Prior gain of function studies using exogenous *IG20*-SVs showed that MADD and *IG20*-SV2 have little or no effect on cell proliferation and susceptibility to induced apoptosis. However, *IG20pa* rendered cells highly susceptible to apoptosis induced by different death signals including TRAIL, and suppressed cell proliferation. In contrast, it is found that DENN-SV was over expressed in tumor tissues and cancer cell lines, and expression of exogenous DENN-SV confers resistance to apoptosis and enhance cell proliferation.

[00051] Down-modulation of select combinations of *IG20*-SVs using siRNAs is demonstrated herein. Synthetic siRNA duplexes or expressed shRNAs can bind to the target mRNA and lead to its degradation. Specific and the most effective shRNAs against *IG20*-SVs were identified by screening 5 different shRNAs targeting all isoforms, and 2 each targeting exons 13L and 16 (FIG. 1A) and cloned into a lentivirus vector. Use of lentivirus resulted in stable expression of shRNAs that is detected through GFP expression. Expressed shRNA down-modulated the targeted *IG20*-SVs (FIG. 2) as early as 24 hours post transduction and lasted at least for 15 days.

[00052] Significant increase in spontaneous apoptosis of HeLa cells with knock-down of all *IG20*-SVs was noted when assayed for nuclear condensation and mitochondrial depolarization; hallmarks

of apoptosis. Earlier studies failed to identify the specific *IG20*-SV responsible, for cancer cell survival.

**[00053]** Significant spontaneous apoptosis was observed at 72 hours although the relevant *IG20*-SV transcripts were down-modulated at 24 hours. This is likely due to persistence of pre-formed proteins, although the possibility that this duration is required for either accumulation of apoptotic, or down-modulation of anti-apoptotic, molecules cannot be ruled out.

**[00054]** To determine the requirement of DENN-SV in cancer cell growth and proliferation, exon 1 3L- and 16- specific shRNAs were expressed in HeLa cells. Interestingly, significant increase in spontaneous apoptosis was observed when *IG20pa*/MADD, but not when *IG20pa*/*IG20*-SV2, were down-modulated (FIG. 3) without affecting the levels of DENN-SV expression. These observations were further substantiated by the observed caspase activation, including caspase-8 and -9 (FIG.-4), and indicated that abrogation of MADD expression alone can induce spontaneous apoptosis of HeLa cells.

**[00055]** Cancer cells die as a consequence of apoptosis due to prolonged arrest in either G1/S or G2/M phases of cell cycle or due to their inability to replicate. Diminished viability of cells upon Mid- and 13L-shRNA expression (FIG. 5) was not a consequence of defective cell proliferation or perturbed cell cycle progression, but was a direct consequence of spontaneous apoptosis. Microscopic examination revealed similar colony size indicating no significant changes in cell growth due to knock-down of various *IG20*-SVs.

**[00056]** Apoptosis was consistently higher in 13L-treated, relative to Mid-treated, cells (FIG. 3 and 6). However, this difference was obscured when a larger amount of MidshRNA virus was used suggesting that relative to the amount of 13LshRNA required to target *IG20pa*/MADD a larger amount of Mid-shRNA is required to knock-down all 4 SVs.

**[00057]** Unlike HeLa cells that express all 4 *IG20*-SVs, the PA-1 (ovarian carcinoma) cell line expresses predominantly MADD and DENN-SV. This cell line was used to unambiguously demonstrate the role of MADD in promoting cancer cell survival. The results obtained on cell proliferation and cell cycle progression with PA-1 cells were very similar to the observations made in HeLa cells, and thereby supported the finding that MADD but not any of the other three *IG20*-SVs can promote cancer cell survival.

**[00058]** Down-modulation of MADD alone can cause spontaneous apoptosis. However, over-expression of exogenous MADD had no discernible effect on induced apoptosis or cell proliferation. Although the mode of action of MADD is not known, it can bind to death receptors (DRs) and thus might prevent spontaneous oligomerization of DRs that leads to apoptosis. If the endogenous MADD (a pro-survival molecule) was sufficient to prevent DR oligomerization, expression of exogenous MADD might have had little or no effect. On the other hand, either down-modulation of MADD or expression of exogenous *IG20pa* (a pro-apoptotic molecule), which might act as a dominant negative, renders cells susceptible to apoptosis by facilitating DR

oligomerization. In contrast, expression of exogenous DENN-SV (an anti-apoptotic molecule) stabilizes or synergizes MADD and prevent apoptosis. It is interesting to note that while IG20pa enhanced TRAIL-induced apoptosis was accompanied by increased recruitment of death-inducing signaling complex (DISC) and caspase activation; DENN-SV induced resistance was characterized by enhanced NF $\kappa$ B activation. Data presented herein demonstrate the requirement of MADD for cancer cell survival and the clinical implication of selective abrogation of MADD.

**[00059]** Data presented herein also demonstrated that MADD abrogation can lead not only to spontaneous apoptosis but also to enhanced TRAIL-induced apoptosis resulting from caspase-8 activation at the DRs and strongly indicated that MADD can act as a negative regulator of caspase-8 activation in cancer cells.

**[00060]** The levels of expression of DRs and DcRs, or their ligands were unperturbed (FIG. 3). Expression of CrmA, a known inhibitor of caspase-1 and -8, or DN-FADD that competes with endogenous FADD conferred resistance to spontaneous apoptosis (FIG. 6). Increased activation of caspase-8 at the DISC was evident from an increase in the p43/p41 fragments. Caspase-8 activation resulting from MADD abrogation was not accompanied by an increase in the recruitment of FADD or caspase-8 to the DISC (FIG. 8).

**[00061]** Although MADD transcripts are depleted by 24h post-transduction of shRNAs, it takes 72h for spontaneous apoptosis to set in (FIG. 8). This allowed us to determine susceptibility to TRAIL-induced apoptosis of MADD-depleted cells. These results showed that MADD abrogation can cause increased caspase-8 activation at the TRAIL-DISC and result in caspase-3 activation, again, without enhancing the DISC formation (FIGS. 9-16).

**[00062]** Over-expression studies failed to indicate a role for MADD in enhanced cell survival. This would suggest that endogenous MADD might be sufficient to fully exert its function and the effects of exogenous MADD, if any, thus may not be apparent. Similarly, although exogenous IG20pa can enhance induced-apoptosis, IG20pa knock-down failed to confer resistance to TRAIL-induced apoptosis. Since IG20pa can be a part of TRAIL-induced DISC, it may act as a dominant-negative MADD and render cells more susceptible to induced-apoptosis. Similarly, over-expression of DENN-SV enhanced cell proliferation and resistance to apoptosis and expression of DENN-SV in the absence of MADD did not prevent apoptosis. DENN-SV, due to its ability to enhance NF $\kappa$ B-activation, might complement the pro-survival function of MADD and thus, upon over expression, can enhance cell survival and proliferation.

**[00063]** Although the mode of action of MADD is not yet known, it can bind to DRs, but not to FADD or caspase-8, and prevent activation of caspase-8 without affecting DR-FADD or FADD-caspase-8 interactions (FIG. 10). The proximity-induced dimerization model for caspase-8 activation suggests that increased proximity of pro-caspase-8 molecules at the receptor allows them to dimerize and undergo activation. Therefore, MADD sterically hinders caspase-8 homodimerization and/or activation through its interaction with the DRs. It is also possible that MADD association

with the DRs can lead to recruitment of other molecules that can either antagonize caspase-8 (e.g. c-FLIP) or are required for the activation of an alternate survival pathway (e.g. MAPKs) that may counteract caspase-8. Nevertheless, results show that MADD can constitutively bind to DRs, and not to FADD or caspase-8, and prevent caspase-8 activation. The fact that loss of endogenous MADD can induce significant spontaneous apoptosis of cancer cells and also enhance their ability to undergo TRAIL-induced apoptosis makes this a desirable therapeutic target that is useful either alone or in conjunction with TRAIL, to develop novel cancer therapies.

**[00064]** MADD cDNA sequence is available on the GenBank database using accession number: NM\_130470. The interfering RNAs disclosed herein that down-regulate MADD are intended to target any allelic variants and naturally occurring mutants of MADD, and polymorphisms that occur in MADD that are found in a particular segment of the population. In other words, sequences that are highly similar (e.g., about 95% at the amino acid level and about 75% at the nucleic acid level) that represent naturally occurring variations in MADD are within the scope of the disclosure, wherein the shRNAs and siRNAs disclosed herein are capable of down-regulating the expression of such sequences. The shRNA sequences presented herein provide a framework and the specification provides guidance (e.g., FIGS. 18-19) to design additional nucleic acid sequences capable of producing interfering RNAs to down-regulate MADD splice variant. MADD sequences that are about 80% or 90% or 95% similar at the nucleic acid level to the MADD sequence disclosed herein are also down-regulated. Accordingly, nucleic acid sequences that generate shRNAs can be redesigned to accommodate the variations if those variations occur within the target region.

**[00065]** Specific down-regulation of MADD splice variant or inhibition of its activity is also possible by agents such as a small molecule, a peptide nucleic acid, an antibody, inhibitor compound, and synthetic nucleic acids that specifically bind to RNA, RNA/DNA hybrids and down-regulate MADD isoform expression.

**[00066]** Nucleic acid or nucleic acid sequence or polynucleotide or polynucleotide sequence refers to the sequence of a single- or double-stranded DNA or RNA molecule of genomic or synthetic origin, *i.e.*, a polymer of deoxyribonucleotide or ribonucleotide bases, respectively.

**[00067]** An isolated nucleic acid sequence is substantially separated or purified from other nucleic acid sequences with which the nucleic acid is normally associated in the cell of the organism in which the nucleic acid naturally occurs. The term includes nucleic acids that are biochemically purified so as to substantially remove contaminating nucleic acids and other cellular components. The term also includes recombinant nucleic acids and chemically synthesized nucleic acids. The term "substantially purified", as used herein, refers to a molecule separated from other molecules normally associated with it in its native state. For example, a substantially purified molecule is the predominant species present in a preparation, such as, isolated BTI nucleic acid after a PCR. In one embodiment, a siRNA molecule includes a double stranded RNA wherein one strand of the RNA is complimentary to the RNA of MADD splice variant. In another embodiment, a siRNA molecule of

the invention includes a double stranded RNA wherein one strand of the RNA comprises a portion of a sequence of RNA having MADD or IG20 gene splice variant sequence. In another embodiment, a siRNA molecule includes a double stranded RNA wherein both strands of RNA are connected by a non-nucleotide linker. Alternately, a siRNA molecule includes a double stranded RNA wherein both strands of RNA are connected by a nucleotide linker, such as a loop or stem loop structure.

**[00068]** Short hairpin RNA (shRNA) contains complementary sense and antisense sequences of a target gene linked by a loop structure. The target sequence starts as a dsDNA cloned into an expression vector that is transcribed to form an shRNA. In the cytoplasm, shRNA is cleaved by Dicer generating short sequences of RNA that can inhibit gene expression by RNAi. The benefit to shRNA is stable transfection of cell lines and enables a single gene in each cell to be targeted. To construct shRNA vectors, see McIntyre and Fanning (2006), Design and cloning strategies for constructing shRNA expression vectors, *BMC Biotechnol.* 2006; 6: 1.

**[00069]** In one embodiment, a single strand component of a siRNA molecule is from about 14 to about 50 nucleotides in length. In another embodiment, a single strand component of a siRNA molecule is about 15-20, 15-21, 14-25, 16-30 or 20-25 nucleotides in length. In yet another embodiment, a single strand component of a siRNA molecule is about 23 nucleotides in length. In one embodiment, a siRNA molecule is from about 20 to about 40 nucleotides in length.

**[00070]** In an embodiment, an antisense nucleic acid molecule, decoy RNA, dsRNA, siRNA, shRNA, or aptamer, nucleic acids include at least one nucleic acid base modification.

**[00071]** In another embodiment, an antisense nucleic acid molecule, decoy RNA, dsRNA, siRNA, shRNA, or aptamer, nucleic acids of the invention comprises at least one phosphate backbone modification.

**[00072]** Methods for treatment of cancer are described wherein cancer includes for example breast cancer, lung cancer, prostate cancer, colorectal cancer, brain cancer, esophageal cancer, stomach cancer, bladder cancer, pancreatic cancer, cervical cancer, head and neck cancer, ovarian cancer, melanoma, lymphoma, glioma, or multidrug resistant cancer, comprising administering to a subject, a nucleic acid molecule or antisense nucleic acid molecule or other nucleic acid molecule capable of down-regulating MADD expression or other splice variant of *IG20* gene under conditions suitable for said treatment.

**[00073]** In another embodiment, conventional or other known drug therapies to be used along with the down-regulation of MADD or prior to or after MADD down-regulation include monoclonal antibodies, specific inhibitors, chemotherapy, or radiation therapy or a combination thereof for cancer.

**[00074]** Specific chemotherapy include paclitaxel, docetaxel, cisplatin, methotrexate, cyclophosphamide, 5-fluoro uridine, Leucovorin, Irinotecan (CAMPTOSAR.™ or CPT-11 or Camptothecin-11 or Campto), Paclitaxel, Carboplatin, doxorubicin, fluorouracil carboplatin,

edatrexate, gemcitabine, or vinorelbine or a combination thereof can be administered along with agents capable of down-regulating MADD or prior to or after down-regulation of MADD.

**[00075]** Antisense nucleic acid refers to a nucleic acid molecule that binds to target RNA by means of RNA--RNA or RNA-DNA or RNA-PNA (peptide nucleic acid) interactions and alters the activity of the target RNA. Typically, antisense molecules are complementary to a target sequence along a single contiguous sequence of the antisense molecule. However, in certain embodiments, an antisense molecule can bind to substrate such that the substrate molecule forms a loop, and/or an antisense molecule can bind such that the antisense molecule forms a loop. Thus, the antisense molecule can be complementary to two (or even more) non-contiguous substrate sequences or two (or even more) non-contiguous sequence portions of an antisense molecule can be complementary to a target sequence or both. In addition, antisense DNA can be used to target RNA by means of DNA-RNA interactions, thereby activating RNase H, which digests the target RNA in the duplex. The antisense oligonucleotides can comprise one or more RNase H activating region, which is capable of activating RNase H cleavage of a target RNA. Antisense DNA can be synthesized chemically or expressed via the use of a single stranded DNA expression vector or equivalent thereof.

**[00076]** Double stranded RNA or dsRNA refers to a double stranded RNA that matches a predetermined gene sequence that is capable of activating cellular enzymes that degrade the corresponding messenger RNA transcripts of the gene. These dsRNAs are referred to as short intervening RNA (siRNA) and can be used to inhibit gene expression. The term "double stranded RNA" or "dsRNA" as used herein refers to a double stranded RNA molecule capable of RNA interference "RNAi", including short interfering RNA "siRNA".

**[00077]** Splice variant refers to a specific isoform of *IG20* gene that is expressed in one or more cell type by alternate splicing.

**[00078]** Consisting essentially of means that the nucleic acid molecule includes a nucleic acid sequence capable of down-regulating a specific splice variant of *IG20* gene. Other sequences can be present which do not interfere with the activity.

**[00079]** In another aspect nucleic acid molecules or antisense molecules that interact with target RNA molecules and down-regulate MADD activity are expressed from transcription units inserted into DNA or RNA vectors. The recombinant vectors are preferably DNA plasmids or viral vectors. Enzymatic nucleic acid molecule or antisense expressing viral vectors can be constructed based on adeno-associated virus, retrovirus, adenovirus, or alphavirus. Preferably, the recombinant vectors capable of expressing the nucleic acid molecules are delivered as described herein, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of shRNA/siRNA/anti-sense nucleic acid molecules. Such vectors can be repeatedly administered as necessary. Once expressed, the interfering nucleic acid molecules bind to the target RNA and down-regulate its function or expression. Delivery of nucleic acid molecule or antisense expressing vectors can be systemic, such as by intravenous or intramuscular administration, by administration to target

cells explanted from the patient or subject followed by reintroduction into the patient or subject, or by any other means that would allow for introduction into the desired target cell. Antisense DNA can also be expressed via the use of a single stranded DNA intracellular expression vector.

**[00080]** Nucleic acid molecules can be administered to cells by a variety of methods known to those familiar to the art, including, but not limited to, encapsulation in liposomes, by iontophoresis, or by an incorporation into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres. Alternatively, the nucleic acid/vehicle combination is locally delivered by direct injection or by use of an infusion pump. Other approaches include the use of various transport and carrier systems, for example, through the use of conjugates and biodegradable polymers.

**[00081]** The molecules disclosed herein can be used as pharmaceutical agents. Pharmaceutical agents prevent, inhibit the occurrence, or treat (alleviate a symptom to some extent, preferably all of the symptoms) of a disease state in a subject.

**[00082]** The negatively charged polynucleotides can be administered (e.g., RNA, DNA or protein) and introduced into a subject by any standard means, with or without stabilizers, buffers, and the like, to form a pharmaceutical composition. When it is desired to use a liposome delivery mechanism, standard protocols for formation of liposomes can be followed. The compositions can also be formulated and used as tablets, capsules or elixirs for oral administration; suppositories for rectal administration; sterile solutions; suspensions for injectable administration; and the other compositions known in the art.

**[00083]** Pharmaceutically acceptable formulations of the compounds are described. These formulations include salts of the above compounds, e.g., acid addition salts, for example, salts of hydrochloric, hydrobromic, acetic acid, and benzene sulfonic acid.

**[00084]** A pharmacological composition or formulation refers to a composition or formulation in a form suitable for administration, e.g., systemic administration, into a cell or subject, preferably a human. Suitable forms, in part, depend upon the use or the route of entry, for example oral, transdermal, or by injection. Such forms should not prevent the composition or formulation from reaching a target cell (i.e., a cell to which the negatively charged polymer is desired to be delivered to). For example, pharmacological compositions injected into the blood stream should be soluble. Other factors are known in the art, and include considerations such as toxicity and forms which prevent the composition or formulation from exerting its effect.

**[00085]** Systemic administration refers to in vivo systemic absorption or accumulation of drugs in the blood stream followed by distribution throughout the entire body. Administration routes which lead to systemic absorption include, without limitations: intravenous, subcutaneous, intraperitoneal, inhalation, oral, intrapulmonary and intramuscular. Each of these administration routes expose the desired negatively charged polymers, e.g., nucleic acids, to an accessible diseased tissue, e.g., tumor tissue. The use of a liposome or other drug carrier comprising the compounds of the instant



invention can potentially localize the drug, for example, in certain tissue types. A liposome formulation which can facilitate the association of drug with the surface of cells, such as, lymphocytes and macrophages is also useful. This approach can provide enhanced delivery of the drug to target cells by taking advantage of the specificity of macrophage and lymphocyte immune recognition of abnormal cells, such as cancer cells.

**[00086]** The nucleic acid molecules disclosed herein and formulations thereof can be administered orally, topically, parenterally, by inhalation or spray or rectally in dosage unit formulations containing conventional non-toxic pharmaceutically acceptable carriers, adjuvants and vehicles. The term parenteral as used herein includes percutaneous, subcutaneous, intravascular (e.g., intravenous), intramuscular, or intrathecal injection or infusion techniques and the like. In addition, there is provided a pharmaceutical formulation comprising a nucleic acid molecule and a pharmaceutically acceptable carrier. One or more nucleic acid molecules can be present in association with one or more non-toxic pharmaceutically acceptable carriers and/or diluents and/or adjuvants, and if desired other active ingredients. The pharmaceutical compositions containing nucleic acid molecules of the invention can be in a form suitable for oral use, for example, as tablets, troches, lozenges, aqueous or oily suspensions, dispersible powders or granules, emulsion, hard or soft capsules, or syrups or elixirs. Pharmaceutical compositions that consist essentially of the nucleic acid sequences to knock-down a specific IG20 splice variant may include about 0.1  $\mu$ g to 1 mg of nucleic acid per kg of body weight. Variations in dosage and effects can be optimized using skills known to a skilled artisan.

**[00087]** In another aspect nucleic acid molecules include an expression vector that includes nucleic acid sequence encoding at least one of the nucleic acid molecules, in a manner which allows expression of that nucleic acid molecule. The expression vector comprises in one embodiment; a) a transcription initiation region; b) a transcription termination region; c) a nucleic acid sequence encoding at least one said nucleic acid molecule; and wherein the sequence is operably linked to said initiation region and said termination region, in a manner which allows expression and/or delivery of said nucleic acid molecule.

### EXAMPLES

**[00088]** The following examples are to be considered as exemplary and not restrictive or limiting in character and that all changes and modifications that come within the spirit of the disclosure are desired to be protected.

**[00089]** **Example 1: siRNAs can selectively knock-down expression of exogenous IG20-SVs.** In order to determine which of the IG20-SVs contribute to apoptosis and cell proliferation, siRNA (small inhibitory RNA) approach was used to selectively knockdown IG20-SVs (Table 1). The siRNAs were cloned into the pSUPER vector to allow for expression of shRNAs (small hairpin RNAs). An aspect of such a vector is shown in FIG. 17. 293T cells were co-transfected with YFP-IG20pa, YFP-MADD or YFP-DENN-SV along with different shRNA-expressing pSUPER

vectors at ratios of 1:1, 1:3 and 1:7. Knock-down of protein expression was assessed by a reduction in YFP expression using flow cytometry. Several shRNAs were screened and results for the most effective ones are shown in FIG. 1A. The 13L-shRNA that targets exon 13L down-modulated IG20pa/MADD, leaving the expression of IG20- SV2 and DENN-SV unaltered. In contrast, 16E-shRNA that targets exon 16, down-modulated IG20pa/IG20-SV2, but not MADD and DENN-SV. The Mid-shRNA targeted at exon 15 down-modulated all *IG20*-SVs. Vector alone or a control shRNA had little or no effect on the expression of *IG20*-SVs. The sequences provided in Table 1 are exemplary and other sequences that specifically target MADD or any other isoform can be designed based on the information given in Table 1 and the disclosure in general.

[00090] The 13L- or 16E-shRNAs were highly specific and had little or no effect on *IG20*-SVs devoid of the targeted exon. To further confirm specificity of Mid-shRNA, silent mutations were created in cDNAs encoding *IG20*-SVs at sites corresponding to the 5<sup>th</sup>, 7<sup>th</sup>, 11<sup>th</sup> and 14<sup>th</sup> nucleotides of the Mid-shRNA. These mutations neither affected the amino acid sequence nor protein expression. Mutant-*IG20*-SV constructs were cotransfected with the pSUPER-shRNA and assessed for their expression. Mid-shRNA failed to down-modulate the mutant-proteins (FIG.1B) further indicating the high specificity of the shRNA to the intended target. FIG. 18 is a schematic illustration of the targets of some of the shRNAs used to knock-down specific *IG20* splice variants (isoforms). FIG. 19 provides schematic illustration of screening for shRNAs (A) and lentivirus production that contain the shRNA expressing vectors (B). Fluorescence values were measured from cells transfected with the respective shRNAs (FIG. 20).

[00091] **Example 2: shRNAs effectively knock-down endogenous *IG20*-SVs.** The shRNAs described herein were cloned into a self-inactivating lentivirus vector (pNL-SINGFP) and generated SUP (vector control), Mid, 13L, 16E and SCR (negative control shRNA) constructs to target specific combinations of endogenous *IG20*-SVs. An illustration of such a vector is shown in FIG. 17. This enabled identification of expression of double copy cassettes likely resulting in enhanced silencing. The transduction efficiency was over 80% as determined by GFP expression. Relative to controls (SUP and SCR) HeLa cells expressing Mid-shRNA showed decrease in all *IG20*-SVs (FIG. 2A). While, 13L-shRNA caused near complete abrogation of only IG20pa and MADD, 16E-shRNA decreased expression of only IG20pa and IG20-SV2 (2A and 2B), without affecting the expression of the other variants. This was confirmed using 13L-forward and B2-reverse primers (FIG. 2C and 2D), and F2-forward and 16E-reverse primers, that amplify only IG20pa and MADD, and not DENN-SV. The effects of loss of various endogenous *IG20*-SV transcripts on the ability of cancer cells to survive were investigated.

[00092] **Example 3: Down-modulation of *IG20*-SVs in HeLa cells leads to spontaneous apoptosis.** Spontaneous cell death was determined by nuclear condensation (Hoechst 33342) and loss of mitochondrial membrane potential (TMRM staining) (FIG. 3A-B). Down-modulation of all *IG20*-SVs using the Mid-shRNA resulted in significant spontaneous apoptosis. While down-modulation

of IG20pa and IG20-SV2 had no effect, abrogation of MADD and IG20pa led to increased spontaneous apoptosis similar to the levels observed in Mid-shRNA expressing HeLa cells. Whether the underlying cause of increased spontaneous apoptosis was similar to that noted after induction of apoptosis by the extrinsic (caspase-8 activation) or the intrinsic (caspase-9 activation) pathway was analyzed by determining the percentage of cells that harbored active caspases 8 and 9 using flow cytometry.

**[00093] Example 4: Abrogation of MADD and IG20pa leads to increased caspase activation.**

Using a pan-caspase inhibitor, a general increase in caspase activation was observed in cells that had reduced MADD expression (FIG. 4A). Increased levels of both active-caspases-8 and -9 were detected in cells treated with either Mid- or 13LshRNA as early as 60 hours (FIG. 4B and C), and peaked 72 hours, post-shRNA transduction; indicating activation of both extrinsic and intrinsic apoptotic pathways in cells lacking MADD.

**[00094] Example 5: Down-modulation of IG20-SVs has no apparent effect on HeLa cell proliferation.**

In order to assess the effects on cell growth and proliferation, various shRNA-expressing viable cells were counted. Relative to controls, a significant decrease in the numbers of viable cells expressing Mid- and 13L-shRNA was observed (FIG. 5). The cells were stained with CFSE and cell division was monitored. Lack of difference in CFSE-dilution with time between the control, Mid- and 13L-shRNA treated cells indicated that the differences in cell numbers were not due to decreased proliferation but spontaneous cell death. Further confirmation was obtained by plating equal numbers of HeLa cells expressing various shRNAs and determining the number and size of colonies after 12 days. Although significantly fewer colonies were formed by Mid- and 13LshRNA expressing cells, the size of the colonies however were comparable to that of controls. Further, cell cycle analysis of shRNA-treated cells failed to show significant differences and indicated that the primary effect of MADD abrogation is spontaneous apoptosis.

**[00095] Example 6: MADD knock-down in PA-1 ovarian carcinoma cells results in spontaneous apoptosis.**

In HeLa cells, down-modulation of all four, or a combination of IG20pa/MADD or IG20pa/IG20-SV2 variants was demonstrated. To unequivocally demonstrate that MADD is required for cell survival, PA-1 ovarian carcinoma cells were used that essentially express only MADD and DENN-SV and thereby facilitate exclusive down-modulation of MADD. While treatment with Mid-shRNA down modulated both MADD and DENN-SV, treatment with 13L-shRNA abrogated only MADD expression (FIG.-6). Further confirmation was obtained by using a different set of primers to amplify MADD and IG20pa (FIG. 6B). Seventy two hours post-transduction with 1 3L-shRNA 50% of the cells underwent spontaneous apoptosis.

**[00096]** The ability of cells to proliferate was also determined. Equal numbers of PA-1 cells (24 hr post-transduction) were cultured and the number of viable cells determined at various time points (FIG. 7A). As seen in HeLa cells, loss of MADD resulted in a significant drop in cell numbers. There was, however, neither a significant difference in CFSE-dilution nor in the size of colonies (FIG.

7B-G). These results clearly demonstrate that endogenous MADD protects cancer cells from spontaneous apoptosis; however, it does not affect their ability to proliferate.

[00097] **Example 7: MADD abrogation results in spontaneous as well as enhanced TRAIL-induced apoptosis without affecting expression of death (DR) or decoy receptors (DcR), or their cognate ligands.** Down modulation of IG20 transcripts upon treatment of cells with lentiviruses expressing different shRNAs was monitored by RT-PCR. The IG20 transcripts were significantly down-modulated by 24h (FIG. 8A, C). These cells were tested for spontaneous apoptosis at 72h post-transduction by Hoechst staining (FIG. 8B, D) or mitochondrial depolarization. Abrogation of MADD, but not other isoforms, resulted in spontaneous apoptosis.

[00098] Although the IG20 transcripts are significantly down-modulated by 24 hrs, the cells do not undergo spontaneous apoptosis until 72 hours post shRNA induction. This is most likely due to the time required for complete degradation of the remaining endogenous proteins. Therefore, at thirty-six hours post-shRNA transduction when there is no indication of spontaneous apoptosis, HeLa cells were treated with various concentrations of TRAIL for different durations and assayed for apoptosis. Cells devoid of MADD showed enhanced TRAIL-induced apoptosis as indicated by significant increases in caspase-3 activation (FIG. 9A, B) and mitochondrial depolarization (FIG. 9C, D). Similar results were obtained in PA-1 ovarian carcinoma cells.

[00099] Since increases in the levels of expression of DRs and their ligands, or decreases in DcRs can result in oligomerization of death receptors followed by apoptotic cell death, the levels of their expression in HeLa and PA-1 cells were tested. No significant difference in the levels of expression in various sh-RNA transduced cells relative to control cells was observed, and indicated that spontaneous apoptosis resulting from MADD down-modulation was not due to perturbations in the levels of DR4, DR5, Fas, FasL, TRAIL, DcR1 and DcR2 expression on the cell surface. Surface expression of DRs, DcRs, and their ligands are not altered upon MADD abrogation. HeLa cells transduced with the indicated shRNAs for 48h were collected in enzyme-free cell dissociation buffer and stained with antibodies (specific to DR4, DR5, DcR1, DcR2, TRAIL, Fas and FasL or isotype controls) conjugated to PE.

[000100] **Example 8: Down-modulation of MADD results in activation of caspase-8 at the DRs that can be inhibited by DN-FADD and CrmA.** Only MADD-depleted HeLa and PA-1 cells (Mid and 13L cells) showed higher amounts of the intermediate active-caspase-8 proteins (p43/41) (FIG. 10A, B). These results indicated that as early as 48h after MADD abrogation, pro-caspase 8 (p55/53) is processed and cleaved into its intermediate (p43/41) active fragments. Similarly, HeLa cells treated with 100ng of TRAIL for the indicated durations (FIG. 11) showed sustained caspase-8 activation and subsequent caspase-3 activation as evident from the increased levels of p43/p41 and p18 subunits of caspase-8, and a concomitant decrease in pro-caspase-3 levels only in MADD-depleted cells.

**[000101]** Further evidence in support of caspase-8 activation in apoptosis induced by MADD abrogation was gained from experiments carried out with HeLa and PA-1 cell lines stably expressing DN-FADD (Dominant-negative FADD) or CrmA. The spontaneous cell death that occurs due to abrogation of endogenous MADD is dramatically inhibited by both CrmA and DN-FADD (FIG. 12). These inhibitors of DISC formation also rendered MADD-depleted HeLa cells (36 hours post-ShRNA transduction) resistant to TRAIL-induced apoptosis (FIG. 13).

**[000102]** Since caspase-8 plays an essential role in receptor-mediated apoptosis that is characterized by DISC formation, the status of DISC in cells undergoing apoptosis due to loss of endogenous MADD expression was examined. DR4 and DR5 from HeLa and PA-1 cells respectively were immunoprecipitated and probed for known DISC components (FIG. 14A, B). FADD and pro-caspase-8 were found to be constitutively associated with the DRs in cells with and without MADD abrogation. However, the intermediate cleaved fragments of caspase-8 (p43/p41) were detected only in MADD-depleted cells, which suggested that activation of caspase-8 at the DRs was associated with spontaneous apoptosis.

**[000103]** TRAIL induced DISC was immunoprecipitated from HeLa cells using a TRAIL-specific antibody and subjected to SDS-PAGE followed by western blotting. Staining for various TRAIL-DISC components revealed co-precipitation of DR4, FADD and caspase-8 (FIG. 15). Relative to control cells (SCR), immunoprecipitates from MADD-depleted cells (13L and Mid) showed increased levels of intermediate fragments of caspase-8 (p43/41). This observation correlated with enhanced caspase-8 activity observed earlier in these cells upon TRAIL treatment (FIG. 11).

**[000104]** **Example 9: MADD binds to DR4, but not caspase-8 or FADD.** To examine whether MADD confers resistance to apoptosis by interacting with caspase-8, MADD-YFP were expressed in HeLa cells and immunoprecipitated it using an IG20-specific antibody from lysates of cells that were left either untreated or treated with TRAIL (250ng) for 30 minutes. These proteins were separated by SDS-PAGE and subjected to western blot analysis to probe for DISC components. Interestingly, DR4 but not caspase-8 co-precipitated with MADD in both untreated and TRAIL-treated cells. On the other hand, FADD co-precipitated with caspase-8 upon TRAIL treatment, while MADD and DR4 was not be co-precipitated with caspase-8 under either condition (FIG. 16). The above results demonstrated that MADD may exert its anti-apoptotic effect by directly binding to DR4 but not to caspase-8 or FADD.

**[000105]** **Table 1: Nucleic acid regions targeted for shRNA knock-down analysis.**

siRNA	Region	Targeting Exon	Targeting Isoform
SUP	Vector Control	None	None

Mid	5'GTACCAGCTTCAGTCTTTC-3'	Exon 15	IG20, MADD, SV-2, DSV
13L	5' CGGCGAATCTATGACAATC-3'	Exon 13L	IG20, MADD
16E	5' CTCTAATGGAGATTGTTAC-3'	Exon 16	IG20, SV-2
SCR control-siRNA	5' TTTAACCGTTTACCGGCCT-3'	None	None

## MATERIALS AND METHODS

**[000106]** *Design of siRNA.* The siRNAs used to target all, or a combination of *IG20*-SVs, are shown in Table 1. Several putative siRNA sequences to target *IG20* transcripts were obtained from the Dharmacon (Lafayette, CO). The most suitable sequences were sorted out based on less than 50% GC content, high AU content towards the 3' end and no inverted repeats within the siRNA region (Reynolds *et al.* (2004), *Nat Biotechnol*, 22(3), 326-30.).

**[000107]** *Plasmid Construction.* The siRNAs were cloned into the pSUPER vector using BglII and HindIII sites (Brummelkamp *et al.*, (2002), *Science*, 296(5567), 550-3.) to generate pS-Mid, pS-13L, pS-16E and pS-SCR plasmids. The respective shRNA cassettes (including the H1 RNA promoter and the shRNA) were excised from the pSUPER plasmids using XbaI and ClaI sites and ligated into the pNLSIN-CMV-GFP vector (Lee *et al.*, (2003), *J Virol.* (22), 11964-72.) to generate SUP (vector control), Mid, 13L, 16E and SCR (negative control) lentivirus constructs. The pcTat, pcRev and pHIT/G were gifts from Dr. BR Cullen and Dr. TJ Hope. The IG20pa-YFP plasmid (Ramaswamy *et al.*, (2004), *Oncogene*, 23(36), 6083-94) was used as a backbone to sub-clone MADD-YFP and DENN-SV-YFP plasmids from respective pBKRSV plasmids (Al-Zoubi *et al.*, (2001), *J Biol Chem*, 276 (50), 47202-11.) using MluI and EcoRV sites. The DENN-SV-YFP-mut, MADD-YFP-mut and IG20-YFP-mut constructs were generated using the Quickchange XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Briefly, the primers 5' 'CGGAACCACAGTACAAGCTTTAGCCTCTCAAACCTCACACTGCC3' (forward) and 5' GGCAGTGTGAGGTTTGAGAGGCTAAAGCTTGTACTGTGGTTCCG3' (reverse) were designed to insert silent mutations at four sites in the cDNAs without affecting the amino acid sequence, as shown in bold, and were used to amplify the mutant-YFP-cDNAs. HindIII site in the mutants, generated due to base substitutions, was used to identify positive clones which were subsequently confirmed by sequencing.

- [000108] *Cell Culture.* 293T cells, HeLa cells and PA-1 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, CA) supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 U/mL penicillin-G and 100 µg/mL streptomycin. All cell lines were maintained at 37° C in a humidified chamber with 5.5% CO<sub>2</sub>.
- [000109] *Screening of shRNAs.* IG20-YFP constructs were co-transfected with different pSUPER-shRNA constructs in different ratios (1 : 1, 1 :3, 1 :7) into 293T cells using Calcium phosphate. 24 hours posttransduction, cells were trypsinized, collected and washed twice in cold PBS, and analyzed for YFP expression by flow cytometry using a FACS Calibur (Becton Dickinson, NJ).
- [000110] *Lentivirus production.* Sub-confluent 293T cells grown in 100 mm plates were co-transfected with 10.8 µg of the respective lentivirus vector, 0.6 µg pcRev, 0.6 µg of pcTat and 0.3 µg of pHIT/G using calcium phosphate. Culture medium was replaced 16 hours later, and the supernatant was harvested 40 hours post-transfection and filtered using a 0.45 µm filter. The optimal viral titer for each cell type was determined as the least amount of viral supernatant required to transduce 80% of target cells without apparent cytotoxicity.
- [000111] *RT-PCR.* Total RNA was extracted from 1 x 10<sup>6</sup> transduced cells using Trizol (Invitrogen Life Technologies, Carlsbad, CA), and 1 jig of RNA was used for RT-PCR using the Super-Script One-Step RT-PCR system (Invitrogen Life Technologies, Carlsbad, CA). Briefly, the cDNAs were synthesized at 50°C for 30 minutes followed by incubation at 94°C for 2 minutes. Subsequently, 35 cycles of PCR were carried out with de-naturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 minute. This was followed by a final incubation at 72°C for 7 minutes. The sequences of F2-B2 and GAPDH primers were as published (Al-Zoubi et al., (2001), *J Biol Chem*, 276 (50), 47202-11.). 13L-Forward (5'CGCCGGCGAATCTATGACAAT3') and B2-reverse primers were used to amplify only MADD and IG20pa. The PCR products were then separated on a 2% agarose gel.
- [000112] *Hoechst Staining.* 5 x 10<sup>5</sup> transduced cells were collected and washed in cold PBS. 1 jig/mL Hoechst 33342 and 5 jig/mL Propidium Iodide (Sigma, St. Louis, MO) were used to stain cells for five minutes. Cells with condensed chromatin were analyzed using a BD-LSR (Becton Dickinson, NJ). Highly PI-positive cells which represent necrotic or late-apoptotic cells were excluded from the analysis. Only GFP-positive cells were included in the analysis.
- [000113] *TMRM staining.* 5 x 10<sup>5</sup> transduced cells were collected and washed in cold PBS and then stained with 1 00nM tetramethylrhodamine methyl ester (Molecular Probes, Invitrogen, CA) for 15 minutes at 37°C. Cells were washed with cold PBS and then subjected to FACS analysis using a FACS Calibur. Only GFP-positive (shRNA-expressing) cells were included in the analysis.
- [000114] *Caspase Detection.* 5 x 10<sup>5</sup> transduced cells were collected and washed 3X in cold PBS and then stained with either Red-z-VAD-FMK (pan-caspases), Red-IETD-FMK (active caspase-8) or RedLEHD-FMK (active-caspase-9) (EMD Biosciences Inc.) for 30 minutes at 37°C. Transduced GFP-positive cells were analyzed for active caspase staining using a FACS Calibur.

- [000115]** *Cell Proliferation.* 24 hours post-transduction,  $5 \times 10^5$  HeLa or  $8 \times 10^5$  PA-1 cells were plated into six-well plates. Every other day, cells were collected, washed and stained with trypan blue, and trypan blue-negative viable cells were counted.
- [000116]** *CFSE Dilution Assay.* 24 hours post-transduction,  $5 \times 10^5$  HeLa or  $8 \times 10^5$  PA-1 cells were stained with 2  $\mu$ M SNARF- 1 carboxylic acid, acetate, succinimidyl ester (S-2280 1, Molecular Probes, Invitrogen, CA) for 15 minutes at 37°C. Cells were then washed and either used immediately for FACS analysis or plated into six-well plates. Every other day, cells were collected, washed and CFSE dilution, as an indicator of cell division, was determined by FACS analysis.
- [000117]** *Crystal Violet Staining.*  $5 \times 10^5$  HeLa and  $8 \times 10^5$  PA-1 cells were plated into six-well plates. 24 hours later cells were treated with different shRNA-expressing lentiviruses for 4 hours. Cells were washed and replenished with fresh warm medium. Twelve days later, cells were fixed in ice-cold methanol and stained with crystal violet to assess viability and colony formation.
- [000118]** *Antibodies and other reagents.* The anti-IG20 peptide polyclonal antibody, raised against 3 different peptides from the N-terminal, middle and C-terminal region of IG20, has been previously described (Al-Zoubi et al., (2001), *J Biol Chem*, 276 (50), 47202-11.). Anti-Caspase-8 antibody (C-15) was a gift from Marcus E. Peter (Ben May Institute of Cancer Research, University of Chicago, Chicago). Anti-FADD antibodies were obtained from BD PharMingen, San Diego, CA., and anti-GFP/YFP antibody (JL-8 clone) was purchased from Clontech Palo Alto, CA. Anti-caspase-8 (C-20) for immunoprecipitation, anti-DR5 (IMG 120), anti-DR4 (H-130), anti-caspase-3 (H-277) and anti-DR4 (B-9 monoclonal) antibodies were obtained from Santa Cruz Biotechnology, Inc., Santa Cruz, CA. Anti-TRAIL-biotinylated antibody and recombinant human-TRAIL were obtained from Peprotech Inc., Rocky Hill, NJ. Anti-actin antibody was obtained from Sigma-Aldrich Corp, CA.
- [000119]** Total RNA was extracted from  $1 \times 10^6$  transduced cells using Trizol (Invitrogen Life Technologies, Carlsbad, CA), and 1  $\mu$ g of RNA was used for RT-PCR using the Super-Script One-Step RT-PCR system (Invitrogen Life Technologies, Carlsbad, CA). RT-PCR was carried out using F2-B2 and GAPDH primers. The PCR products were then separated on a 2% agarose gel.
- [000120]** *FACS analysis of cell surface expression of receptors.* Forty-eight hours post-transduction, HeLa cells were collected in enzyme-free cell dissociation buffer (Invitrogen, CA), washed once with PBS containing 0.5% BSA and let stand in the same buffer for 10 minutes at 4° C. PE-conjugated anti-DR4 (DJR1 clone), anti-DR5 (DJR2-4 clone), anti-DcR1 (DJR3 clone), anti-DcR2 (DJR4-1 clone), anti-TRAIL (clone RIK 2) and anti-FasL (NOK1) antibodies purchased from eBiosciences, San Diego, CA and anti-Fas (BD Pharmingen) were used for staining samples for 30 minutes at 4° C. A mouse IgG antibody was used as isotype control. Cells were washed with PBS and GFP-positive cells were analyzed by using a FACS Calibur (Becton Dickinson, NJ).
- [000121]** *Suppression of apoptosis using DN-FADD and CrmA.* HeLa and PA-1 cells were transfected with either DN-FADD, CrmA or control PCDNA 3.1 vector using Super-Fect reagent (Qiagen Inc., CA). Permanently transfected cells were selected in 800  $\mu$ g/mL of G418. Post-



selection, stably transfected cells were grown in medium containing 400 µg/mL of G418. The stable cells were transduced with the respective lentiviruses and 72h post-transduction, cells were assayed for spontaneous apoptosis. Cells were treated with 100ng of TRAIL 36h post-transduction and assayed for apoptosis by active-caspase-3 staining.

**[000122]** *Active-caspase-3 detection by FACS.* Active-caspase-3 levels were detected by analyzing PE-positive population using the active-caspase-3-PE staining kit (BD Pharmingen, San Diego, CA). Only GFP-positive cells were included in the analysis using a FACS Calibur.

**[000123]** *Characterization of DISC immunoprecipitated using anti-DR4/DR5 antibody.* HeLa cells ( $2 \times 10^7$ ) and PA-1 cells ( $5 \times 10^7$ ) transduced for 48h were collected and washed in cold PBS. Washed cells were lysed in 1 mL of lysis buffer [30mM Tris/HCl, pH 7.5, 150mM NaCl, 2mM EDTA, 1mM phenylmethylsulfonyl fluoride (PMSF), protease inhibitors cocktail (Roche, Mannheim, Germany), 1% Triton X-100 and 10% glycerol] on ice for 30 minutes and clarified by centrifugation at 12000 rpm for 30 minutes at 4° C. Supernatants were normalized for protein concentration and then immunoprecipitated using 2µg of H-130 DR4/DR5 antibody on a rotoshaker at 4° C for 4h followed by addition of 25µL of 50% slurry of Protein A/G (Amersham, Piscataway, NJ) beads for 2 hours. The beads were then washed three times with lysis buffer and boiled in SDS lysis buffer (62.5 mM Tris-HCl, 2% SDS, 10% glycerol, 50mM DTT and bromophenol blue, pH 6.8) for 5 minutes. Eluates were then subjected to SDS-PAGE using a 12% gel for subsequent immunoblot analysis.

**[000124]** *Characterization of DISC immunoprecipitated using anti-TRAIL antibody.* HeLa ( $2 \times 10^7$ ) cells transduced for 36h were treated with 250ng of TRAIL (Peprotech) for 30 minutes either at 4° C (untreated) or at 37° C (treated), collected, washed in cold PBS and lysed in 1mL lysis buffer for 30 minutes on ice. The lysates were clarified and then normalized for protein concentration. The DISC was then immunoprecipitated overnight using 2µg/mL of anti-TRAIL-biotinylated antibody. The biotinylated antibody was immunoprecipitated with 35 µL of 50% slurry of streptavidin agarose beads. The beads were washed three times with lysis buffer, boiled in SDS sample buffer and separated on a 12% SDS-PAGE gel for immunoblot analysis.

**[000125]** *Immunoprecipitation of the MADD complex.* HeLa cells were transfected with MADD-YFP. Thirty-six hours post-transduction HeLa ( $2 \times 10^7$ ) cells were collected and left untreated or treated with 250ng/mL TRAIL for 30 minutes at 37° C. At the end of the treatment, cells were washed in cold PBS and pelleted and then lysed in 1mL of DISC lysis buffer for 30 minutes on ice and clarified by centrifugation. The supernatants were normalized for protein concentration, pre-cleared and were incubated with 10 µL of polyclonal anti-IG20 antibodies on a rotoshaker overnight. The complexes were immunoprecipitated with 50% slurry of protein A/G beads. The beads were washed three times and boiled in SDS lysis buffer for 5 minutes. The eluates were then subjected to SDS-PAGE using a 12% gel for immunoblot analysis.

**[000126]** *Immunoblotting.* The membranes were blocked in 5% non-fat dry milk in PBS-Tween (PBS with 0.05% Tween 20) for 1 hour. Primary antibodies were used at a concentration of 1 µg/mL and

the secondary antibodies were used at a 1:10000 concentration. The blots were developed by enhanced chemiluminescence according to the manufacturer's protocol (Pierce Biotechnology Inc., Rockford, IL).

[000127] *Sequences:*

[000128] An shRNA sequence for "mid" siRNA is GTACCAGCTTCAGTCTTTCTTCAAGAGA GAAAGACTGAAGCTGGTAC, with the hairpin loop region highlighted in bold.

[000129] An shRNA sequence for "13L" siRNA is CGGCGAATCTATGACAATCTTCAAGAGA GATTGTCATAGATTGCGCCG, with the hairpin loop region highlighted in bold.

[000130] The cDNA sequence of MADD splice variant is as follows:

[000131] cacgtgcatg ttagcatgc cttggtttt ctttggcat ctgaaaagg cacaacctga aagacctaga acccagtgc  
ggccccagg ccttttggga caggaagaga agagccgtgt ggccgcgggg aggatgtcct gcggcggggc tgcctcgcg  
gactgactgg actccatctc ccagcgggcg ccgcggcgcg gccacgccc cccactccc gcgcgcgccc ggtggagact  
tcgattitca gaattcctcc tgggaatgct gactccttc ttggtgccct gatgcttctc tgagataaac tgatgaattg gaacctggt  
gcaaaagaag aagtctctc ctcggttact tgactatcta gtgacgtag gggccaggca cccgagcagt gatagcgtg  
cccagactcc tgaattgcta cggcgatacc cttggagga tcacactgag ttcccctgc cccagatgt agtgtcttc tgccagccc  
agggtgcct gagggtgcg cagcggcgca tgagcctcg ggatgatacc tctttgtct tcacctcac tgacaaggac  
actggagta cgcgalatgg calctgtgt aactctacc gctcctcca aaagcgaatc tctaaggaga agggggaagg  
tggggcaggg tccgtggga aggaaggaa ccatgccacc tgtgctcag aagaggggtg cactgagagc tcagagagt  
gctatccct gcagccttc agtgcgtact ctaccctga tgtgaaccag tctcctcgg gcaaacgcc ggccaaggcg  
gggagccgt cccgcaacag tactctacg tccctgtcg tctcagcca ctacccttc ttccacct tccgagagt  
ttgtatact ctcaagcgc tgggtgactg ctgtagtag cgcctctgg gcaagaaact gggcatccct cgaggcgtac aaaggacac  
catgtggcgg atcttactg gatcgtgct ggtagaggag aagtcaagt cctcttgc tgacctga gagattgagg cctggatca  
tcgattgtg cgtccccag taccgtctc tgggcagaag cgagtagaca tcgaggtcct accccaagag ctccagccag  
cttgacctt tgccttcca gacctctc gattaccct agtggattc cactgcacc tccccttga actctaggt gtggacgct  
gtccaggt gtaacctgc attctgttag agcacaagg ggtgctacag tccgagact acaatgcact ctccatgtct gtgatggcat  
tcgtggcaat gatctacca ctggaataa tgtttcigt catcccgctg ctaccacct gcatggcacc agcagagcag ctgctgttg  
ctcaacccc gtacatcatt ggggttctg ccagctctt cctctacaaa ctggacttca aaatgcctga tgatgatgg ctagtggatc  
tggacagcaa taggtgatt gccccacca atgcagaagt gctgcctatc ctgccagaac cagaatcact agagctgaaa aagcattaa  
agcaggcctt ggccagcatg agtctcaaca cccagcccat cctcaatctg gagaaattc atgagggcca ggagatcccc  
cttctcttg gaaggcctt taatgacctg cgtccacac cgtccactga atcaaccca ctcalctat gcaatgatgt ggattctgtg  
gatgttcaa ccagggttgc catggtacgg ttcttcaatt ccgcaacgt gctgcaggga ttccagatgc acacgcgtac cctgcgcctc  
ttcctcggc ctgtggtagc tttaagct ggctccttc tagcctcacg tcccggcag actcctttg ccgagaaatt ggccaggact  
caggctgtg agtacttgg ggaatggatc cttaaccca ccaactatgc ctttcagcga attcacaaca atatgtttga tccagccctg  
attgtgaca agccaaagt gtatgctcat cagctgcagc ctatccacta tcgctctat gacagcaatt cccagctggc tgaggccctg  
agtgtaccac cagagcggga ctctgactcc gaacctactg atgatagtgg cagtgatagt atggattatg acgattcaag ctctcttac  
tctccttg gtgacttgt cagtgaatg atgaaatgt acattaatgg tgatactccc aatgtggacc ctctgacaca tgcagcactg  
ggggatgcca gcgaggtgga gattgacgag ctgcagaatc agaaggaagc agaagagcct ggcccagaca gtgagaactc

tcaggaaaac cccccactgc gctccagctc tagcaccaca gccagcagca gcccacgac tgatccac ggagccaact  
 ctgaacctgc tgactctacg gagatggatg ataaggcagc agtagggctc tocaagcccc tccctccgt gccctccagc attggcaaat  
 cgaacatgga cagacgtcag gcagaaatig gagaggggic agtgcccg cgaatctaig acaatccata ctccagcccc  
 caataaggct tccccctga ggaagatgag gatgagcagg gggaaagtta cactccccga ttcagccaac atgtcagtgg  
 caatcgggct caaaagctgc tgcggcccaa cagcttgaga ctggcaagt actcagatgc agagtcagac tctcgggcaa  
 gctctccaa cccacccgct tccaacacca gcaccgaggg ctccgggggc atcatgtct ttcagcag cctctatcgg aaccacagta  
 ccagctcag tcttcaaac ctcaactgc ccacaaaagg tgcagagag aaggccacgc cctccccag tctgaaagga  
 aacaggaggg cgttagtgga tcagaagtca tctgtcata aacacagccc aacagtgaaa agagaaccic catcaccca  
 gggcgcac agcaattcta gtgagaacca gcagtcctg aaggaggtgg tgcacagcgt gctggacggc caggaggtg  
 gctggctcaa catgaaaaag gtgcgccggc tctggagag cagcagctg cagctcttg tctgagcaa gctgaaccgc  
 atggtgcagt cagaggacga tgcggcag gacatcatc cggatgtgga gatcagtcgg aaggtgtaca agggaatgtt  
 agacctctc aagtgtacag tctcagctt ggagcagtc tatgccacg cgggtctggg tggcatggc agcatcttg ggctttgga  
 gattgccag acccactact atagtaaaga accagacaag cggagagaa gtccaacaga aagttaant acccagttg  
 gcaaggatcc tggcctagct gggcgggggg acccaaaggc tatggcaca ctgagagttc cacaactggg acctcgggca  
 ccaagtcca caggaaaggg tctaaggaa ctggacacca gaagtttaa ggaagaaaat ttatagcat ctattgggccc tgaagtaac  
 aaactgtct ttagcttg ttagacagag gagaaaaagt cccagatcag cgcagacagt ggtgtgagcc tgactctag  
 tcccagagg actgatcaag actctgcat cggcgtgagt ccagctgtta tgaaccgag ctcaagtcag gattctgaag ttagcaccgt  
 ggtgagtaat agctctggag agacccttg agctgacagt gacttgagca gcaatgcagg tgatggacca ggtggcagg  
 gcaggttca cctggcaagc tctcggggca ctgtctga tagtgaaat gagaccaact ctgccacaag caccatctt ggtaaagccc  
 acagctgaa gccaagcata aaggagaagc tggcaggcag cccattcgt actctgaag atgtgagcca gcgagctat ctctatagg  
 gactcctagg caaagagcgt tctactttat gggaccnaat gcaattctg gaagatgcct tcttagatgc tgtgatgtg gagagagaag  
 ggatgggtat ggaccagggt cccaggaaa tgaacagag gtacctgtc ctggagaac atgaccggaa gcgcctggaa  
 gatgatgaag atcgttctg gccacacit ctgcacaacc tcatctcta catgtctct atgaaggtaa ataagaatga catccgcaag  
 aaggtaggc gcctaattgg aaagtcgac attgggcttg tgtacagcca gcaaatcaat gaggtgctt atcagctggc gaacctgaat  
 ggacgcgac tctctatctg gtccagtggc agccggcaca tgaagaagca gacatttg gtacatgcag ggacagatc  
 aaacggagat atcttttca tggaggtgtg cgtgactgt gtgtgtgtg gtagtaacat cggaaacagt tatgagcgt ggtgtgacga  
 gaagctcat aacatgacct actgtccaa gacgaagggt ttgtctgtt ggcgtagaaa tggctctgag accagctca acaagtcta  
 tactaaaaag tctcgggagc tgtactact tgtgaaggac agcatggagc gcgctgccg cgcagacaa agcatcaaac  
 ccggacctga attgggtggc gagttccctg tgcaggacct gaagactggg gaggggtggc tctgcaggt gacctggaa  
 gggatcaacc tcaaatcat gcacaatcag gtttcatag agctgaatca caaaaaag tgaatacag ttcaggcgt ctgtctctg  
 gaggaattt tictgaaat taaagaagt gtgagccaca agtaacag accaatggc cagaaatct gctactccgt attatgtct  
 tctctgacg tggctgcagt tcatagcagt gaggaagatc tcagaacccc gcccggcct gctctagct gatggagagg ggctacgag  
 ctgccccagc ccagggcag cccctggccc ctgtgttc ccaagtgcac gatgtctgt tgaatgagga tggatgatg ctgtgtgtc  
 ctctgcaagc cccctgtgt ggcttggtg gttaccggt atgtgtccct ctgagtgtt ctgagcgtg tccacctct cctctccac  
 tccagaaga ccaactgcc tccccctcag ggcacaagaa tgtgtacagt ctgtggggc ggtgtgaacc cactatttg tgccttgag  
 acatttgtt tgtgttct tgtctgtc cctggcgtta taactgtca ctgcaagagt ctggctctc ctctctgtg acccgcatg  
 actgggcgcc tggagcagtt tcatctgtg aggagtggg gaacctggg gctaccctc tcagaggaag ggcacagaga

ggaagggaag aattgggggg cagccggagt gaggggcagc ctccctgctt cttctgcat tcccaagccg gcagctactg  
cccagggccc gcagtgttg ctgctgccig ccacagctc tgtgactgca gtggagcggc gaattccctg tggcctgcc  
cgccttcggc atcagaggat ggagtggtc aggctagtgg agtcccagg accgctggct gcctgcctg agcatcaggg  
agggggcagg aaagaccaag ctgggttgc acatctgtc gcaggctgtc tctccaggca cgggggtgca ggaggagag  
acagcctggg tatgggcaag aatgactgt aaataftca gcccacatt attatagaa aatgtacagt tgtgtgaatg tgaataaat  
gtctcaact ccc

[000132] In the MADD cDNA sequence presented above, the highlighted portion in bold indicates nucleotides that are absent in a variation of the MADD splice variant, whose sequence is provided below:

atggtgcaaaagaagaagtctgtcctcggttacttgactatctagtatcgtaggggccaggcaccgagcagtgatag  
cgtggcccagactcctgaattgctacggcgatacccttggaggatcacactgagttccctgccccagatgtagt  
tctctgccagcccagggtgctgagcgtgcggcagcggcgcatgagccttcgggatgatacctctttgtcttcacc  
ctactgacaaggacactggagtcacgcgatatggcatctgtgttaacttctaccgctccttccaaaagcgaatcttaa  
ggagaagggggaaggtggggcagggtcccgtgggaaggaaggaacccatgccacctgtgcctcagaagagggtggcactg  
agagctcagagagtggctcatccctgcagcctctcagtgtgactctacccctgatgtgaaccagtctcctcggggcaaa  
cgccgggccaagggcggggagccgctcccgaacagtactctcacgtccctgtgcgtgtcagccactacccttcttctc  
caccttcgagagtgtttgtatacttcaagcgcctgggtgactgtgtagtgccttctgggcaagaaactgggca  
tccctcgaggcgtacaaaggacaccatgtggcgatcttactgtagctgtgtagaggagaagtcaagtgcctt  
ctgcatgaccttcgagagattgaggcctggatctatcgattgtgcgtccccagtagccctgtctgggcagaagcgagt  
agacatcgaggctctacccaagagctccagccagctctgaccttctcttccagaccatctcgttacccttagtg  
attcccactgcaccttcccttgaacttctaggtgtggacgctgtctccaggtgctaacctgcattctgttagagcac  
aaggtggtgtacagtcggagactacaatgcactctccatgtctgtatggcattcgtggcaatgatctaccactgga  
atatatgttctgtcatcccgtctacccacctgcatggcatcagcagcagctgtgttggctccaaccccgatca  
tcattgggttcttccagcttcttctacaaaactggacttcaaaatgcctgatgatgtatggctagtggatctggac  
agcaatagggtgattgccccaccaatgcagaagtgtgcctatctgccagaaccagaatcactagagctgaaaaagca  
tttaaagcaggccttggccagcatgagctcaacaccagccatcctcaatctggagaaattcatgagggccaggaga  
tcccccttcttgggaaggccttctaatgacctgcagtccacaccgtccactgaattcaaccactcatctatggcaat  
gatgtggattctgtgatgttgaaccagggttgcctggtacggttctcaattccgccaacgtgctgcagggtattca  
gatgcacacgcgtacctgcgccttcttctcgctgtggtatgctttcaagctggctccttctagcctcacgtccc  
ggcagactccttttgcgagaaattggccaggactcaggctgtggagtactttggggaatggatccttaacccaccaac  
tatgccttccagcgaattcacaacaatatgttgcacagccctgattggtgacaagccaaagtgtgatgctcatcagct  
gcagcctatccactatcgctctatgacagcaattccagctggctgaggccctgagtgtaccaccagagcgggactctg  
actccgaacctactgatgatgtggcagtgatgatgtgattatgacgattcaagctcttctactctcccttgggtgac  
tttgcagtgaatgatgaaatgtgacattaatgtgatactcccaatgtggacctctgacacatgcagcactggggga  
tgccagcgagggtggagattgacgagctgcagaatcagaaggagcagaagagcctggcccagacagtgaactctcagg  
aaaacccccactgctcctcagctctagcaccacagccagcagcagcccccagcactgtcatccacggagccaactctgaa  
cctgctgactctacggagatggatgataaggcagcagtaggcgtctccaaagcccctcccttccgtgcctccagcattgg  
caaatcgaacatggacagacgtcaggcagaaattggagaggggtcagtgccggcggaatctatgacaatccatactcg  
agcccaatatggccttccctgaggaagatgaggatgagcaggggggaaagttaactccccgattcagccaacatgtc  
agtggcaatcgggctcaaaagctgtgcggcccaacagcttgagactggcaagtgactcagatgcagagtcagactctc  
ggcaagctctcccaactccaccgtctccaacaccagcaccgagggttcgggggcatcatgtcttttgcagcagcctct  
atcggaaccacagtaccagcttcttcaaacctcactgcccacaaaaggtgcccagagagaaggccacgccttc  
cccagctgaaagggaacaggaggcggttagtgatcagaagtcattgtcattaaacacagcccaacagtgaagagaga  
acctcatcaccacagggtcgatccagcaattctagtgaacacagcagttcctgaaggagggtgtgcacagcgtgctgg  
acggccagggtggtggtcgaacatgaaaaggtgcggcgtgctggagagcagcagctgcgagcttcttctcctg  
agcaagctgaaccgcatggtgagtcagaggacgatcccgagcagcatcatccggatgtggagatcagtcggaaggt  
gtacaagggaatgttagacctctcaagtgacagtcctcagcttggagcagtcctatgccacgcgggtctgggtggca

tggccagcatctttgggcttttgagattgccagaccactactatagtaaagaaccagacaagcgggaagagaagtcca  
 acagaaagtgtaaataccccagttggcaaggatcctggcctagctgggcggggggacccaaaggctatggcacaactgag  
 agttccacaactgggacctcgggcaccaagtgccacaggaaagggtcctaaggaaactggacaccagaagttaaagggaag  
 aaaaatttatagcatctattgggcctgaagtaatacaaacctgtctttgaccttgggtgagacagaggagaaaaagtcccag  
 atcagcgcagacagtgggtgagcctgacgtctagtcccagaggactgatcaagactctgtcatcggcgtgagtcacgc  
 tgttatgatccgcagctcaagtgcaggattctgaagtgaataagctctggagagacccttggagctg  
 acagtgactgagcagcaatgcagggtgatggaccaggtggcgaggggcagtggtcacctggcaagctctcggggcactttg  
 tctgafagtgaattgagaccaactctgccacaagcaccatcttggtaaagcccacagctgaagccaagcataaagga  
 gaagctggcagcagccccattcgtacttctgaagatgtgagccagcagtgctatctctatgagggactcctaggcaag  
 agcgttctactttatgggaccaaattctgggaagatgccttcttagatgctgtgatgttgagagagaagggatg  
 ggtatggaccagggtccccaggaatgatcgacaggtacctgtcccttgagaacatgaccggaagcgcctggaagatga  
 tgaagatcgttggcgcacacttctgcacaacctcatctcctacatgctgctgatgaaggtaaataagaatgacatcc  
 gcaagaagggtgagggcgccaatgggaaagtcgcacattgggcttgtgtacagccagcaaatcaatgaggtgcttgatcag  
 ctggcgaaacctgaatggacgcgatctctctatctggtccagtggcagccggcacatgaagaagcagacatttgggtaca  
 tgcagggacagatacaaacggagatacttttcatggaggtgtgcgatgactgtgtgtgttgcgtagtaacatcgga  
 cagtgtatgagcgtggtgtgtacgagaagctcatcaaatgacctactgtcccaagacgaaggtgttgtgcttggcgt  
 agaatggctctgagaccagctcaacaagtctatactaaaaaggtgtcgggagctgtactactgtgtgaaggacagcat  
 ggagcgcgtgccgcccagacagcaagcatcaaacccggacctgaattgggtggcgagttccctgtgcaggacctgaaga  
 ctggtgagggtggcctgtgcaggtgaccttggaaaggatcaacctcaaatcatgcacaatcaggtttcatagagctg  
 aatcacattaaaaagtgaatacagttcagggcgtcttgcctggaggaaattgttccctgaaattaaagagtggtgag  
 ccacaagtacaagacaccaatggcccacgaaatctgctactccgtattatgtctcttctctgtacgtggctgcagttcata  
 gcagtgagggaagatctcagaaccccgccccggcctgtctctagctga

[000133] The amino acid sequence of MADD is as follows:

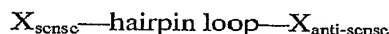
[000134] MVQKKKFCPRLLDYLIVVGARHPSSDSVAQTPELLRRYPLEDHTEFPLPPDVVFFCQ  
 PEGCLSVRQRRMSLRDDTSFVFTLTDKDTGVTRYGICVNFYRSFQKRISKEKGEGGAGSRG  
 KEGTHATCASEEGGTESSESGLSLQPLSADSTPDVNQSPRGKRRAKAGSRSRNSTLTSCLVL  
 SHYFFSTFRECLYTLKRLVDCCSERLLGKKLGIPRGVQRDTMWRIFTGSLVVEEKSSALLH  
 DLREIEAWIYRLLRSPVPVSGQKRVDIEVLPQELQPALTFALPDPSRFTLVDFPLHPLLELLG  
 VDACLQVLTCLLEHKVVLQSRDYNALSMSVMAFVAMIYPLEYMFVPIPLPTCMASAEQL  
 LLAPTPYIIGVPASFLLYKLDFKMPDDVWLVDLDSNRVIAPTNAEVLPIPEPESELEKHLK  
 QALASMSLNTQPILNLEKFHEGQEIPLLGRPSNDLQSTPSTEFNPLIYGNDVDSVDVATRVA  
 MVRFFNSANVLQGFQMHTRTLRLFRPVVAFQAGSFLASRPQTPFAEKLARTQAVEYFGE  
 WILNPTNYAFQRIHNNMFDPALIGDKPKWYAHQLQPIHYRVYDSNSQLAEALSVPPEPDS  
 SEPTDDSGSDSMDYDDSSSSYSSLGDFVSEMMKCDINGDTPNVDPLTHAALGDASEVEIDE  
 LQNQKEAEPPGPDSENSQENPPLRSSSTTASSSPSTVIHGANSSEPADSTEMDDKAAVGVSK  
 PLPSVPPSIGKSNMDRRQAEIGEGSVRRRIYDNPFEPQYGFPEEDEDEQGESYTPRFSQHV  
 SGNRAQKLLRPNSLRLASDSAESDSRASSPNSTVSNSTSTEGFGGIMSFASSLYRNHSTSFSL  
 SNLTLPTKGAREKATPFPSLKGNNRRALVDQKSSVIKHSPTVKREPPSPQGRSSNSSENQQLK  
 EVVHSVLDGQGVGWLNMKKVRRILLESEQLRVFVLSKLNRMVQSEDDARQDIIPDVEISRK  
 VYKGMILLKCTVLSLEQSYAHAGLGGMASIFGLLEIAQTHYYSKEPDKRKRSPTESVNTF  
 VGKDPGLAGRGDPKAMAQLRVPQLGPRAPSATGKGPKELDTRSLKEENFIASIGPEVIKPVF  
 DLGETEEKKSQISADSGVSLTSSSQRTDQDSVIGVSPAVMIRSSSQDSEVSTVVSNSSETLG

ADSDLSSNAGDGPGEVHSLASSRGTLSDSEIETNSATSTIFGKAHSLKPSIKEKLAGSPIRT  
SEDVSQRVYLYEGLLGKERSTLWDQMQFWEDAFLDAVMLEREGMGMDQGPQEMIDRYL  
SLGEHDRKRLEDDEDRLATLLHNLISYMLLMKVNKNDIRKKVRRLMGKSHIGLVYSQQIN  
EVLDQLANLNGRDLSIWSSGSRHMKKQTFVVHAGTDTNGDIFFMEVCDDCVVLRNIGTV  
YERWWYEKLINMTYCPKTKVLCLWRRNGSETQLNKFYTKKCRELYYCVKDSMERAAAR  
QQSIKPGPELGGEFPVQDLKTGEGGLLQVTLEGINLKFMHNQVFIELNHIKKCNTVRGVFVL  
EEFVPEIKEVVSHKYKTPMAHEICYSVLCLFSYVAAVHSSEEDLRTPPRPVSS.

## CLAIMS:

1. An isolated nucleic acid that selectively down-regulates the expression of a splice variant of an *IG20* (Insulinoma-Glucagonoma) gene, wherein the splice variant is MADD.
2. Use of the nucleic acid of claim 1 to specifically down-regulating the expression of a splice variant of an *IG20* (Insulinoma-Glucagonoma) gene comprising:
  - (a) obtaining the nucleic acid molecule of claim 1, wherein the nucleic acid molecule or a transcription product thereof is capable of selectively binding to a RNA molecule, the RNA molecule comprising a nucleic acid sequence of a MADD splice variant of the *IG20* gene; and
  - (b) contacting a cell that expresses the MADD splice variant of the *IG20* gene with the nucleic acid molecule, wherein the nucleic acid molecule down-regulates the expression of the MADD splice variant.
3. The use of claim 2, wherein the nucleic acid molecule is selected from the group consisting of siRNA, shRNA and anti-sense molecule against the MADD splice variant of *IG20*.
4. The use of claim 2, wherein the nucleic acid molecule targets exon 13L of the MADD splice variant.
5. The use of claim 2, wherein the nucleic acid molecule comprises a nucleotide sequence of CGGCGAATCTATGACAATC or a transcribed product thereof, sufficient to knock-down the expression of MADD splice variant or an allelic variant or a mutant thereof.
6. Use of the nucleic acid of claim 1 to inhibit the growth of a cancer cell, the method comprising:
  - (a) obtaining the nucleic acid molecule of claim 1, wherein the nucleic acid molecule is capable of selectively binding to a RNA molecule of a MADD splice variant of the *IG20* gene; and
  - (b) contacting a cancer cell that expresses the MADD splice variant of the *IG20* gene with the nucleic acid molecule, wherein the nucleic acid molecule down-regulates the expression of the MADD splice variant.
7. The use of claim 6, wherein the nucleic acid molecule is selected from the group consisting of siRNA, shRNA and anti-sense molecule against the MADD splice variant of *IG20* that targets a nucleotide sequence of MADD selected from the group comprising CGGCGAATCTATGACAATC, allelic variation thereof, polymorphisms thereof, and a genetic mutation thereof.
8. Use of the nucleic acid of claim 1 to regulate the growth of a cancer cell, the method comprising:
  - (a) administering a pharmaceutical composition consisting essentially of the nucleic acid of claim 1 that specifically down-regulates the expression of a MADD splice variant of the *IG20* gene in a cancer cell; and
  - (b) exposing the cancer cell to a cancer treatment selected from the group consisting of radiation therapy, chemotherapy, and antibody therapy or a combination thereof.
9. Use of the nucleic acid of claim 1 to increase apoptotic cell death in a cancer cell, the method comprising:
  - (a) administering the nucleic acid molecule of claim 1, the nucleic acid capable of specifically down-regulating the expression of a MADD splice variant of an *IG20* gene in a cancer cell; and

- (b) increasing the apoptotic cell death in the cancer cell.
10. The use of claim 9, wherein the apoptotic cell death is effected by a caspase.
  11. The use of claim 9, wherein the apoptotic cell death is induced by a TNF-related apoptosis inducing ligand (TRAIL).
  12. The use of claim 9, wherein the nucleic acid molecule comprises a nucleotide sequence CGGCGAATCTATGACAATC or an RNA equivalent thereof.
  13. A method of selectively inhibiting a splice variant of an *IG20* (Insulinoma-Glucagonoma) gene, the method comprising:
    - (a) obtaining an agent that selectively inhibits the expression of an *IG20* splice variant; and
    - (b) contacting a cell that expresses the splice variant to inhibit the splice variant.
  14. The method of claim 13, wherein the splice variant is MADD.
  15. The method of claim 13, wherein the agent is a molecule selected from the group consisting of siRNA, shRNA and an anti-sense molecule against the *IG20* splice variant.
  16. A pharmaceutical composition consisting essentially of the nucleic acid of claim 1 capable of selectively inhibiting the expression of a MADD splice variant in a cancer cell.
  17. The pharmaceutical composition of claim 16, wherein the nucleic acid sequence comprises CGGCGAATCTATGACAATC or an RNA equivalent thereof.
  18. A vector comprising the nucleic acid of claim 1 capable of selectively inhibiting the expression of a MADD splice variant in a cancer cell, wherein the nucleic acid comprises CGGCGAATCTATGACAATC.
  19. A cell comprising the nucleic acid molecule of claim 1, wherein the nucleic acid specifically down-regulates the expression of a MADD splice variant of an *IG20* gene.
  20. The nucleic acid of claim 1, wherein the nucleic acid encodes a short interfering RNA, the nucleic acid comprising the structure:



wherein X comprises a nucleic acid sequence CGGCGAATCTATGACAATC.

21. The nucleic acid of claim 20, wherein the nucleic acid sequence is CGGCGAATCTATGACAATCTTCAAGAGAGATTGTCATAGATTCGCCG, wherein the hairpin loop region is from positions 20-28.
22. The nucleic acid of claim 1 is synthetic.
23. The nucleic acid of claim 1 encoding an RNA molecule comprising a nucleic acid sequence CGGCGAAUCUAUGACAAUC.



1/18

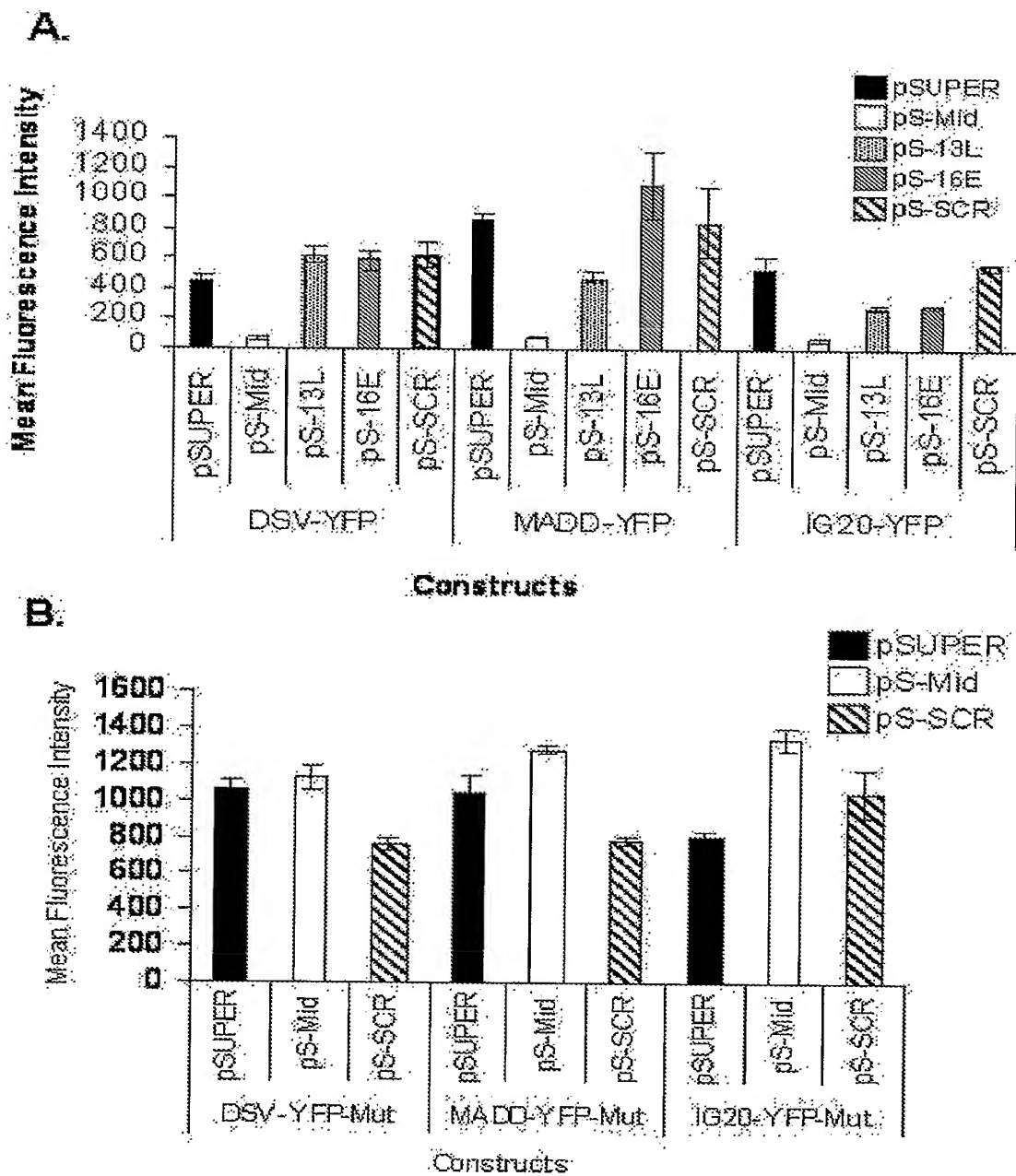


FIG. 1

2/18

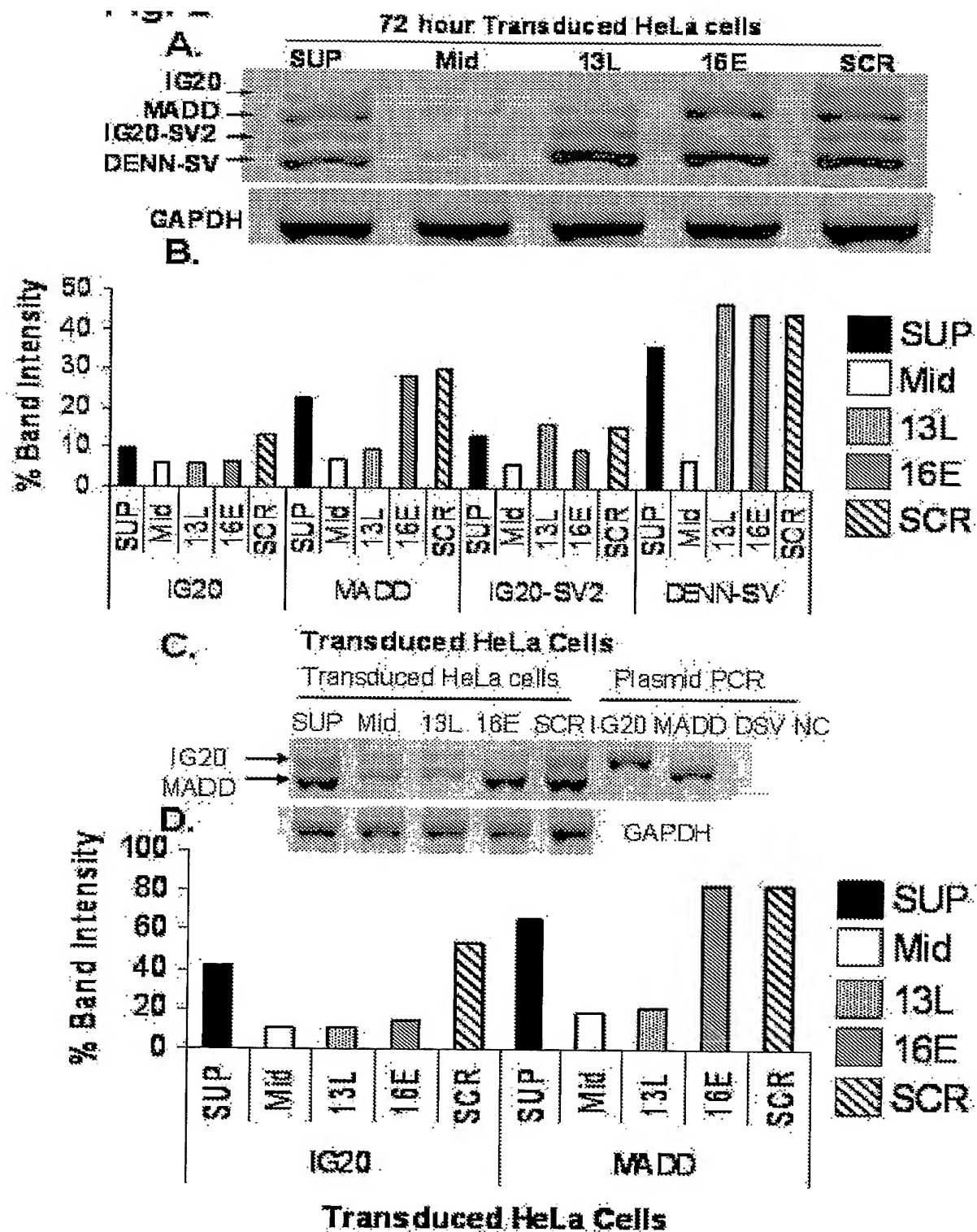


FIG. 2

3/18

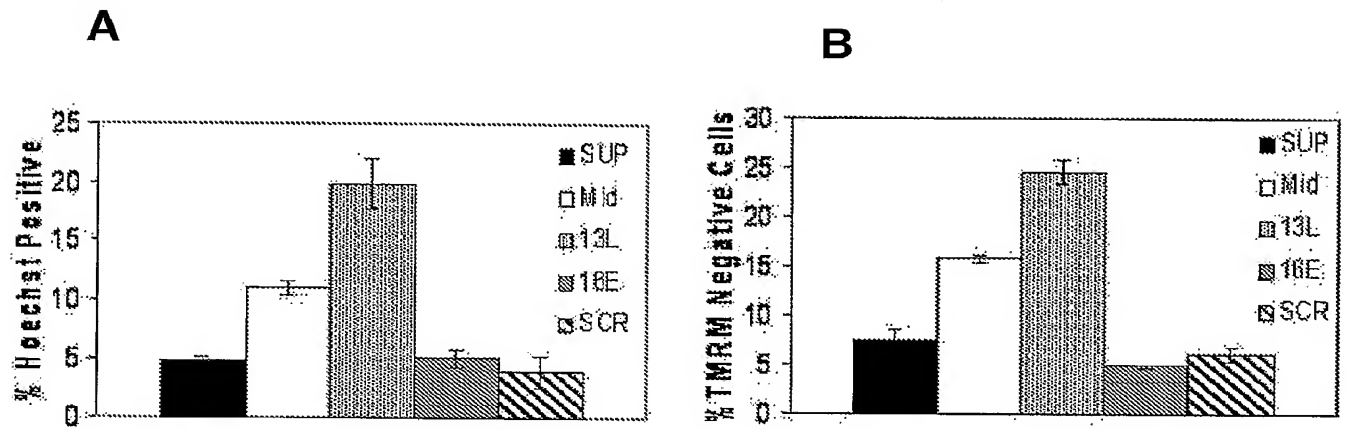


FIG. 3

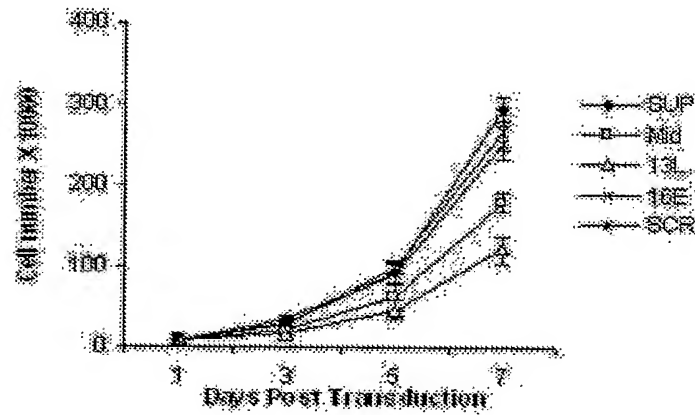
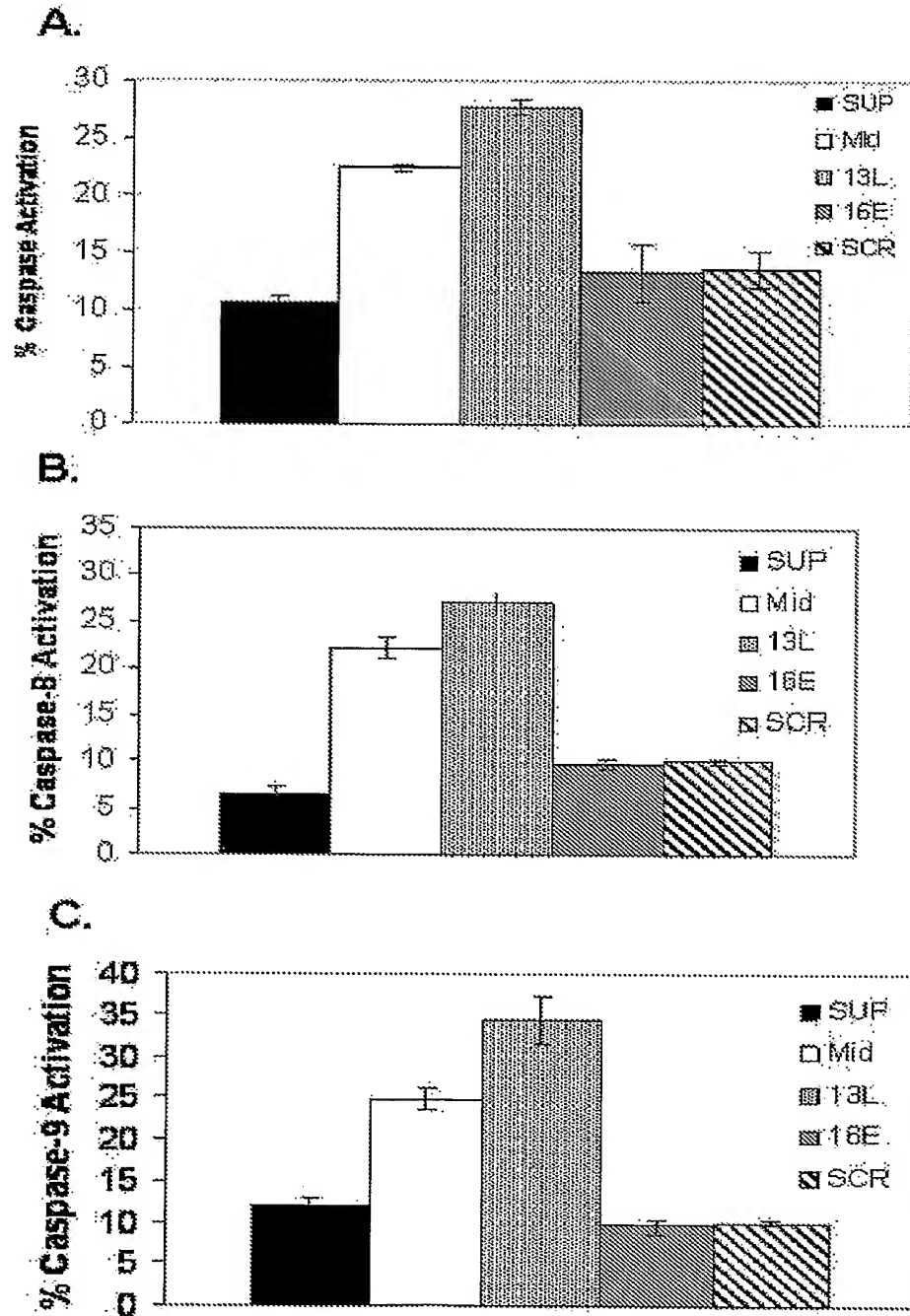


FIG. 5

4/18

**FIG. 4**

5/18

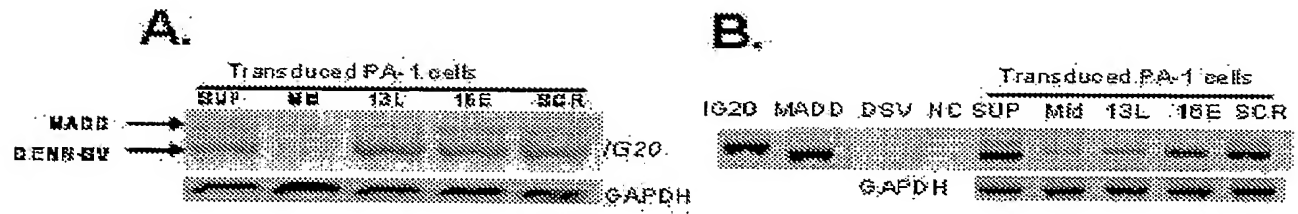


FIG. 6

6/18

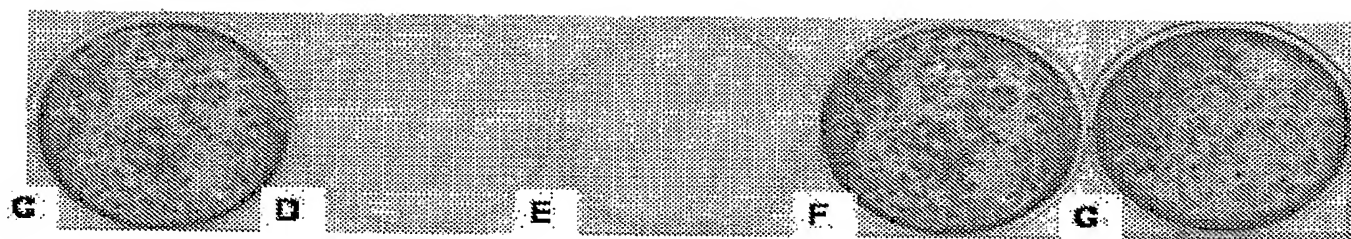
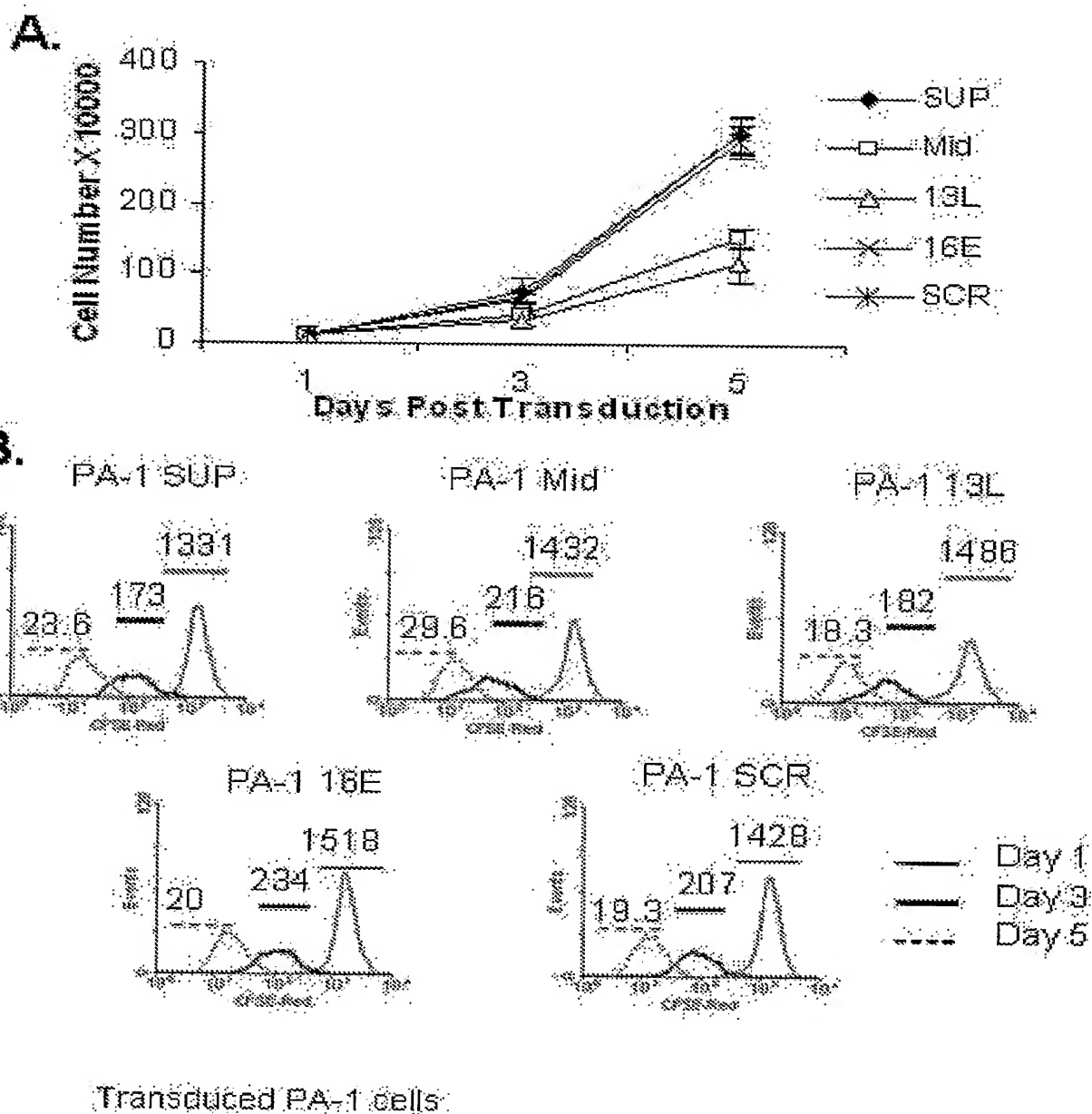
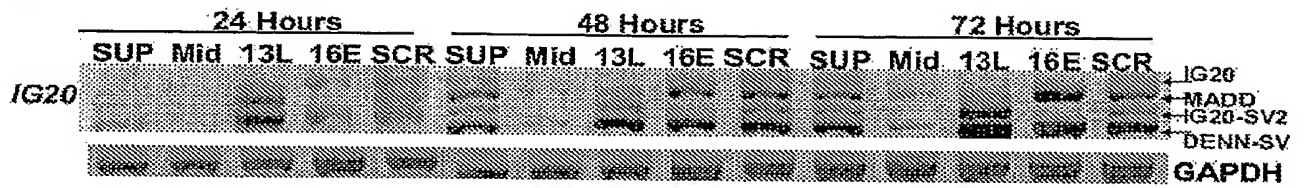
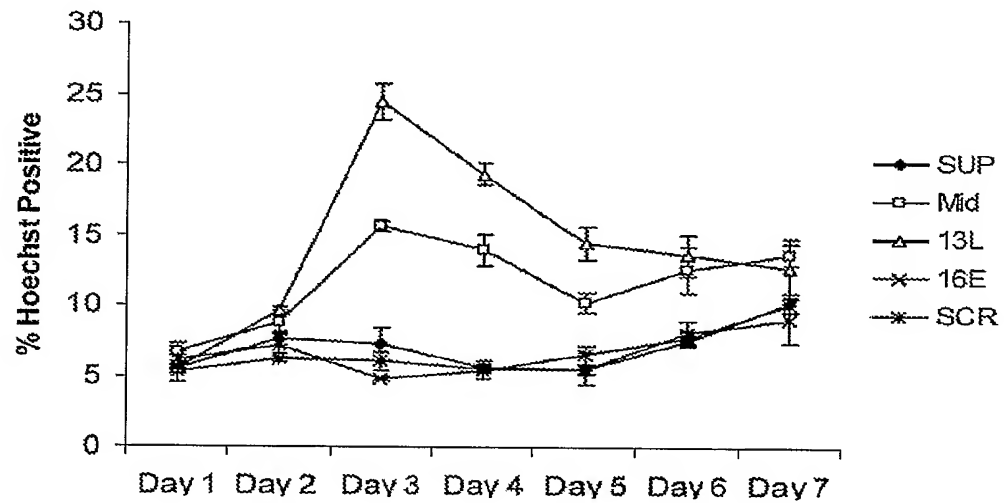
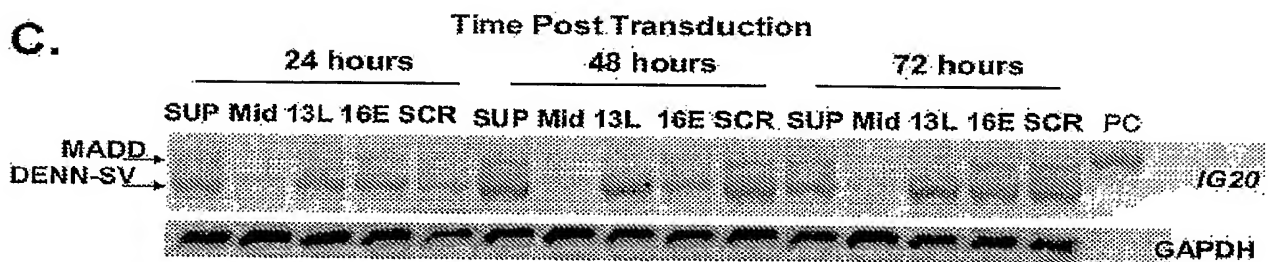
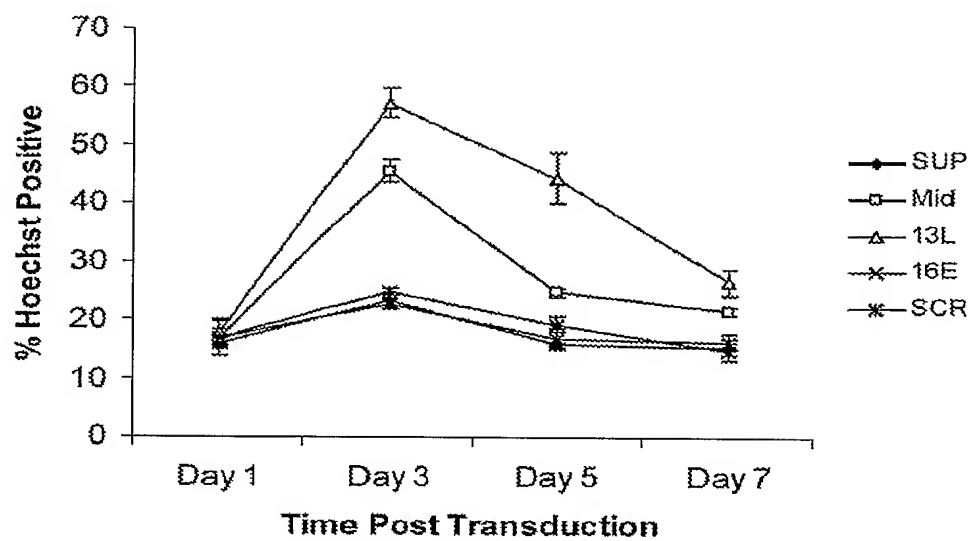


FIG. 7

7/18

**A.****B.****C.****FIG. 8A-C**

8/18

**D.****FIG. 8D**



9/18

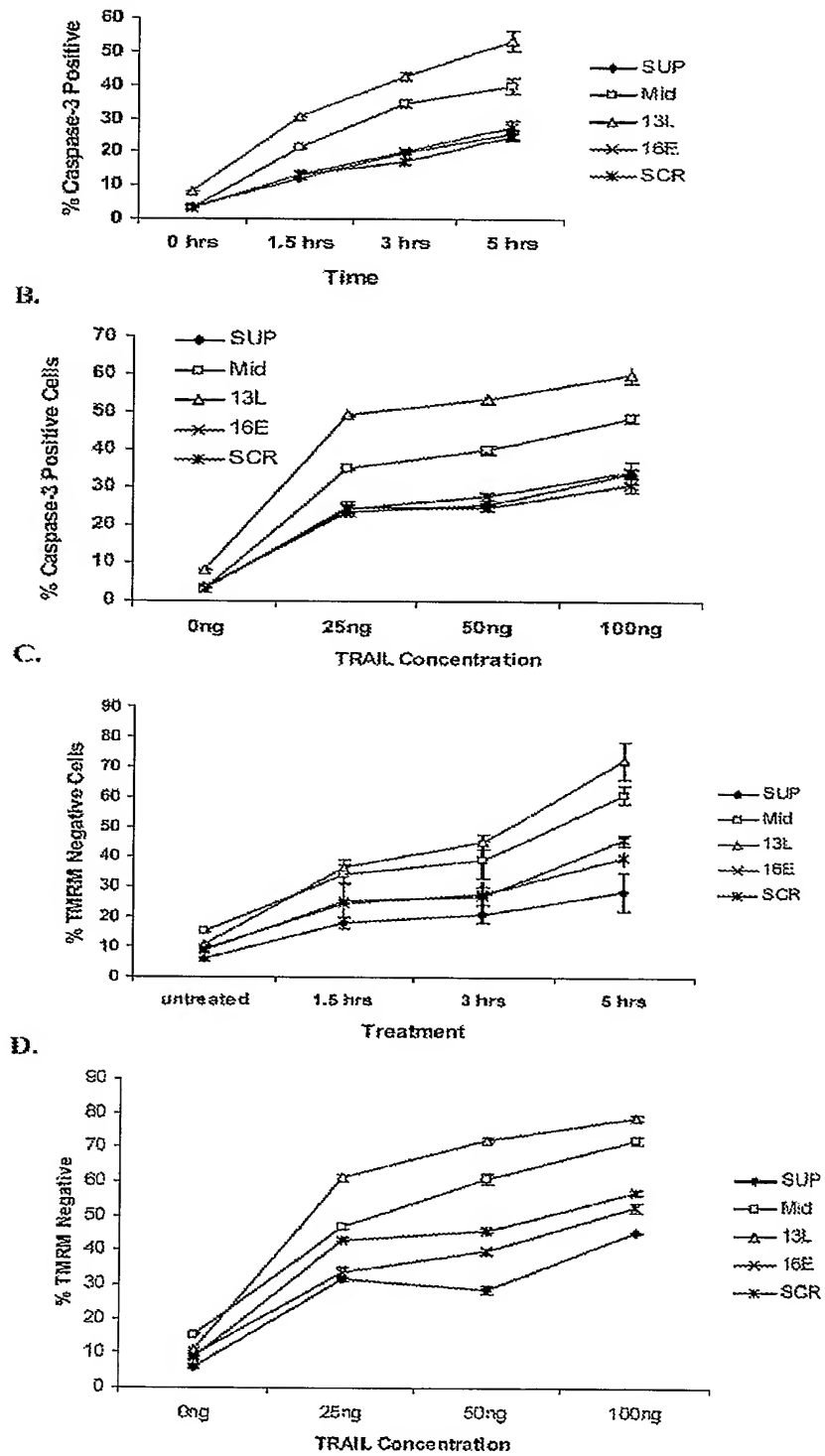


FIG. 9

10/18

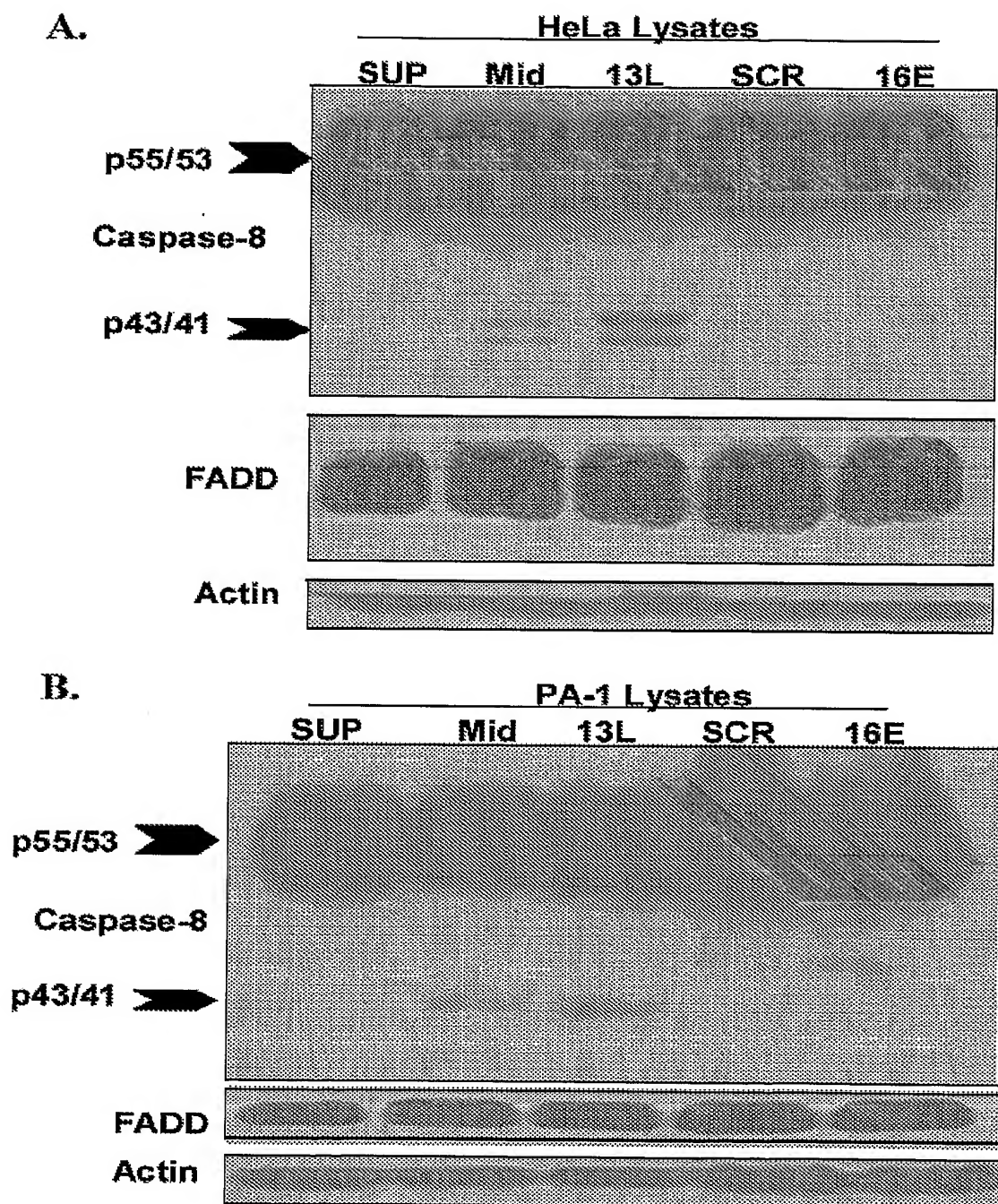


FIG. 10

11/18

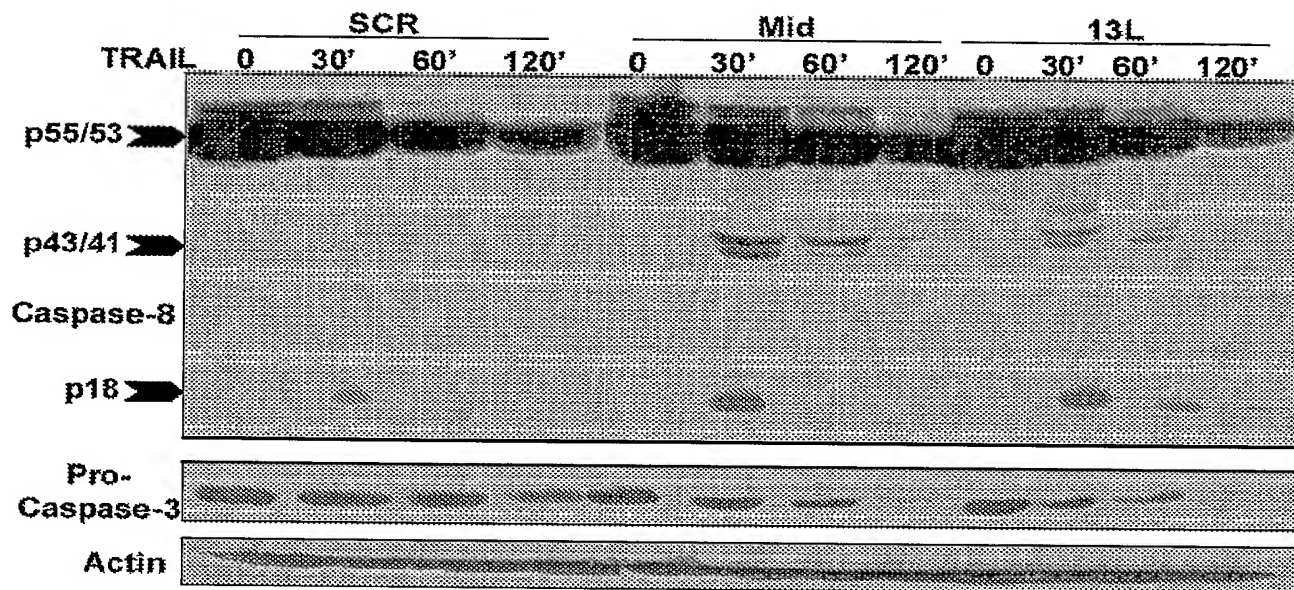
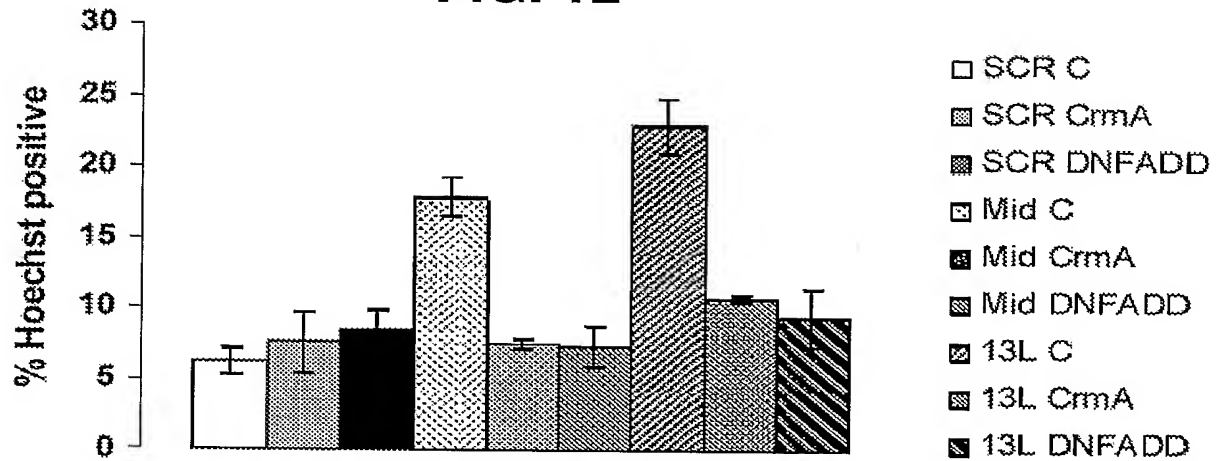


FIG. 11

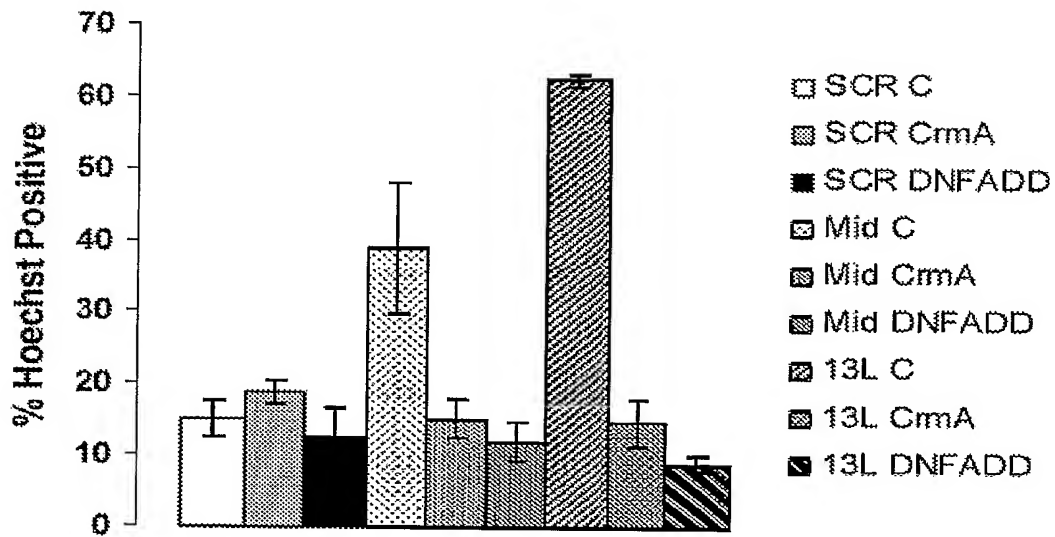
12/18

A.

FIG. 12



B.



13/18

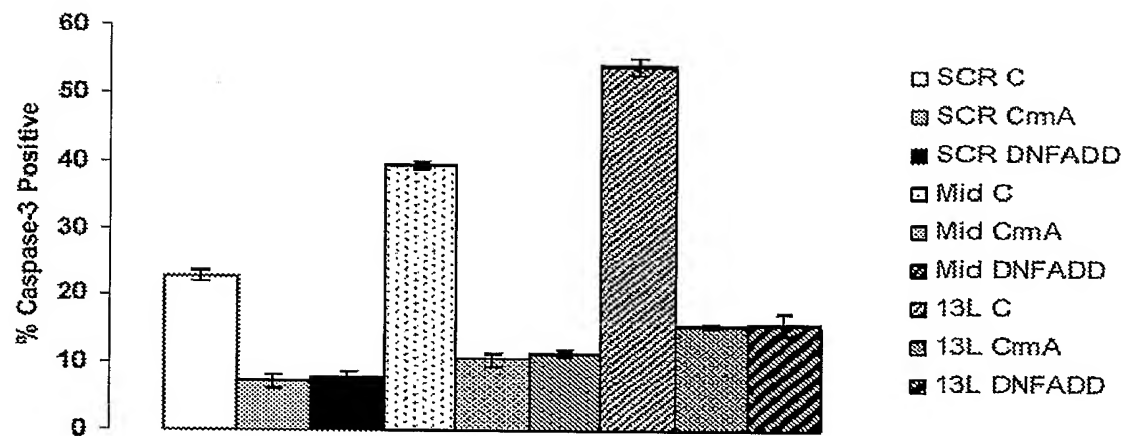


FIG. 13

14/18

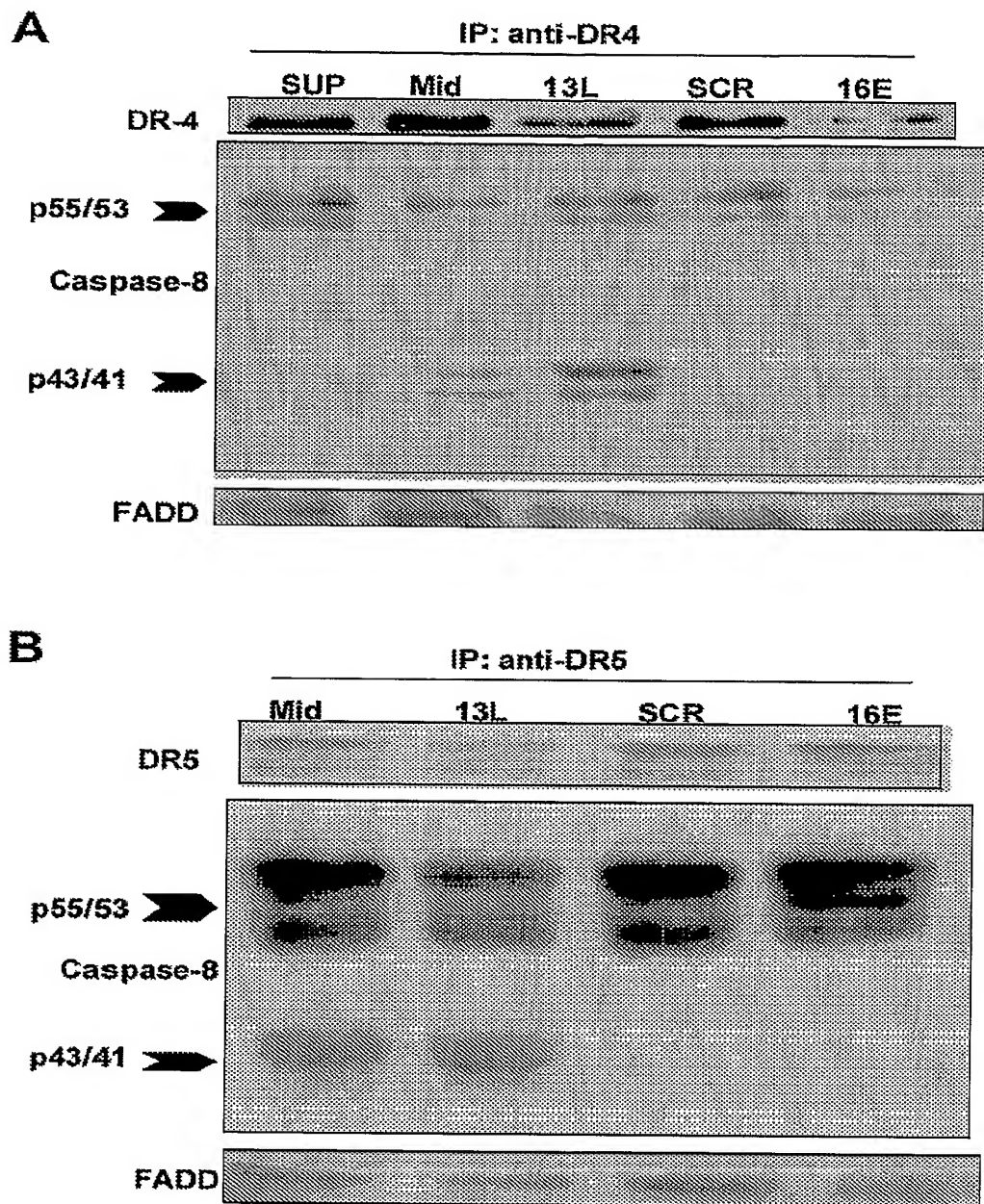


FIG. 14

15/18

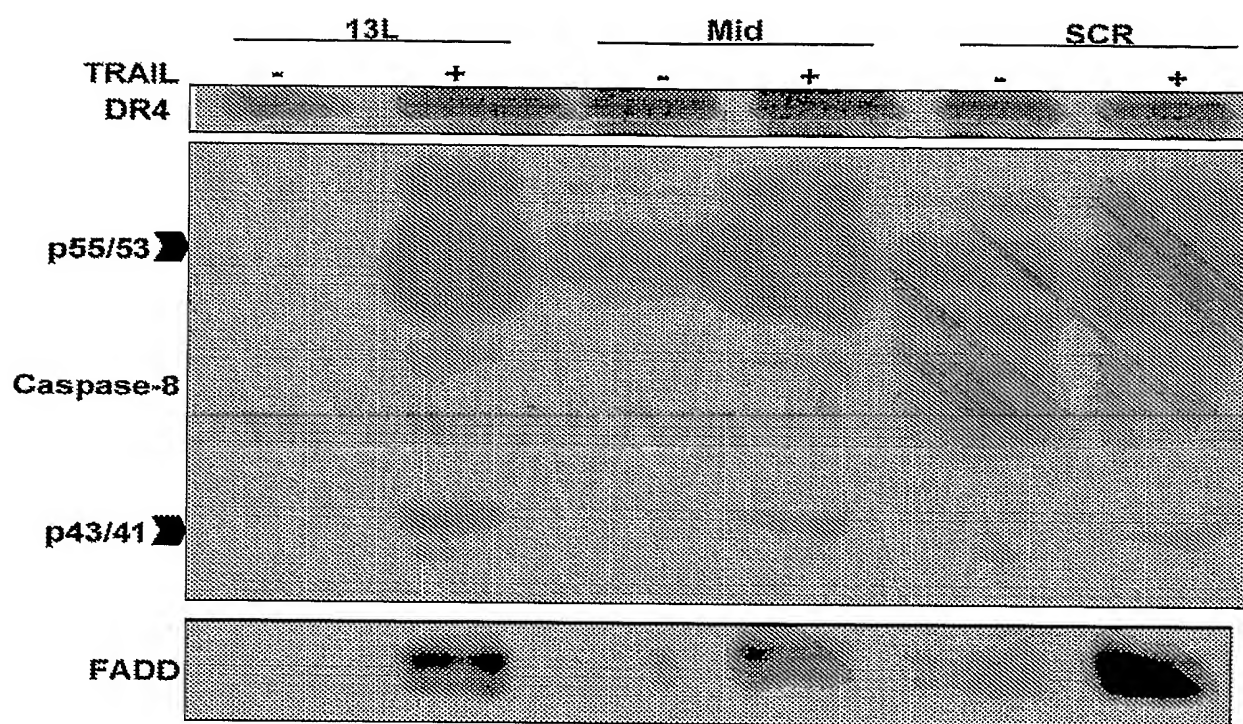


FIG. 15

16/18

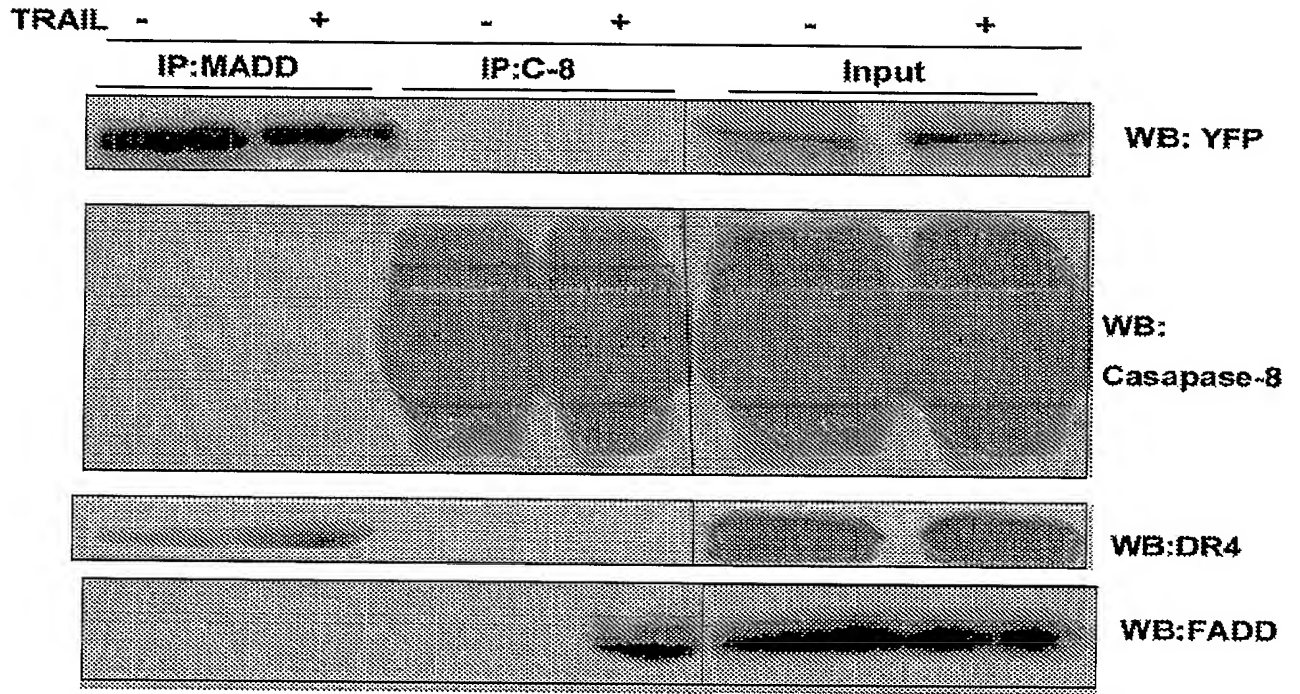


FIG. 16

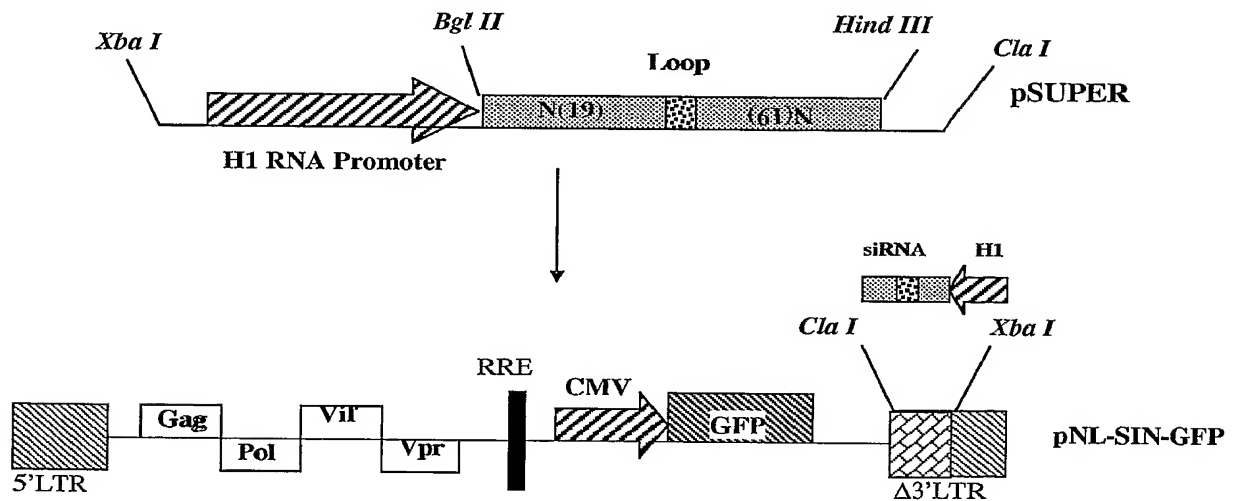


FIG. 17



17/18

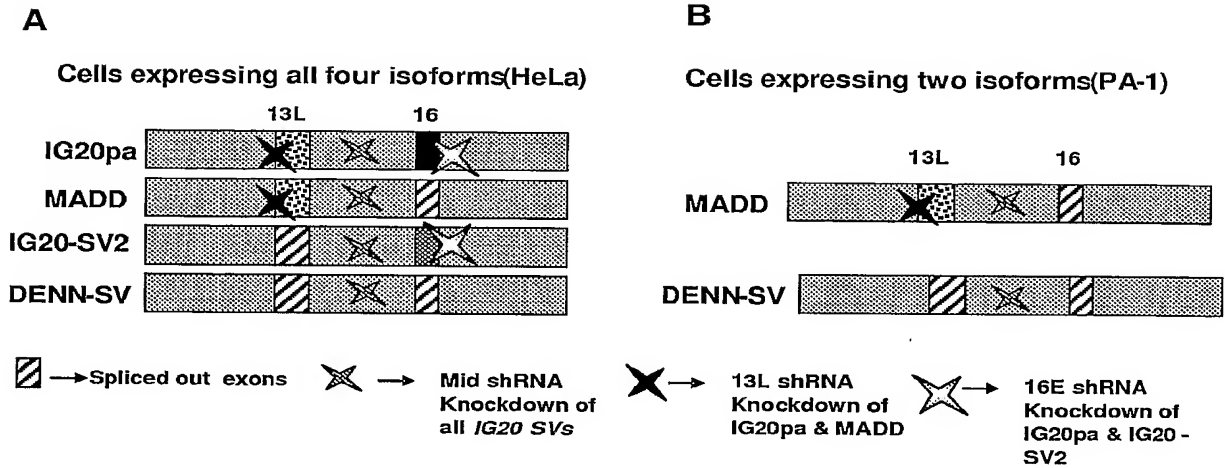


FIG. 18

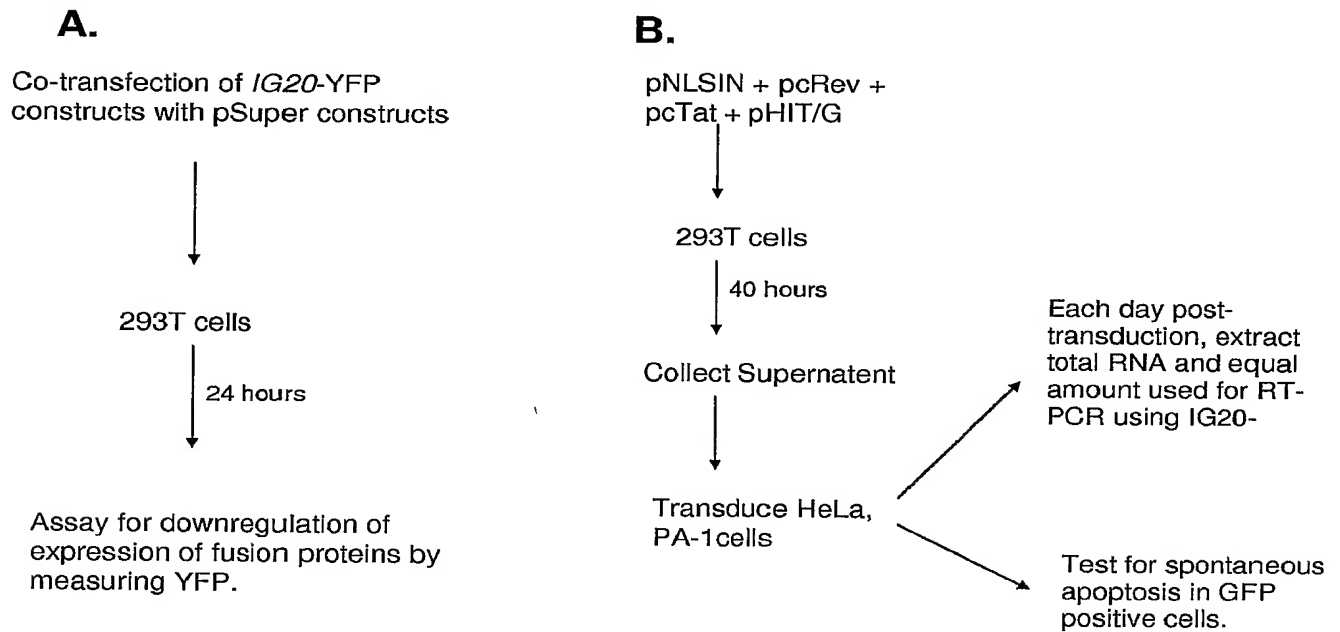
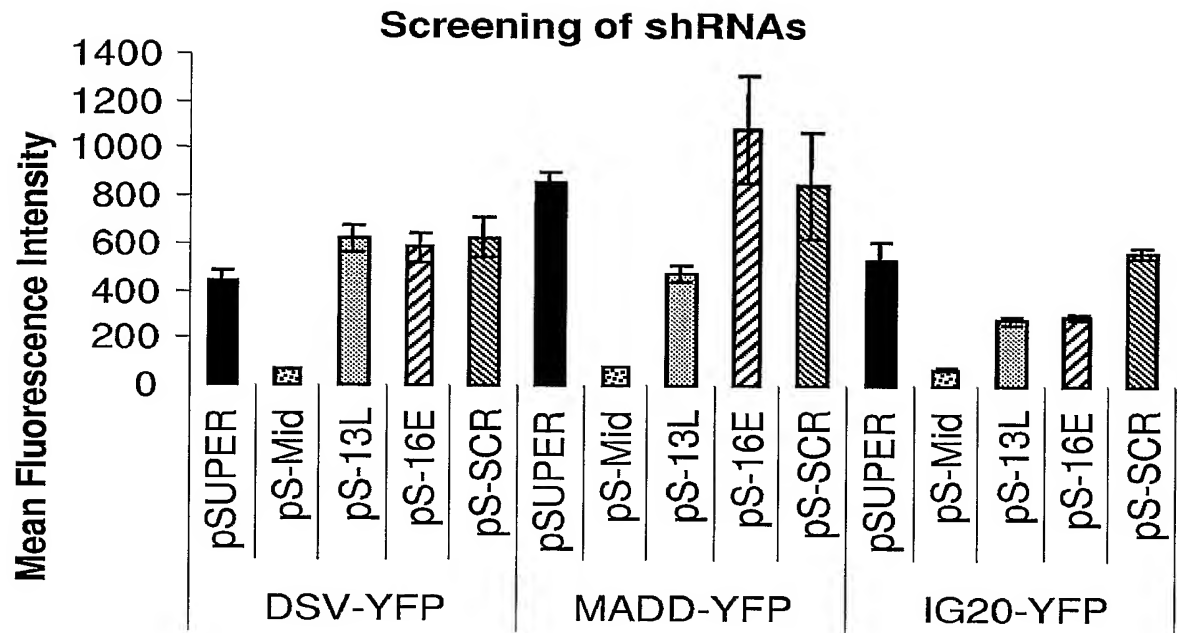


FIG. 19

18/18

**FIG. 20**